Biotransformation of the Analgesic-Antipyretic Drugs Metamizole and Aminopyrine by Genetically Polymorphic Enzymes

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To my parents

And my children Omran and Raian

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
4-AA	4-aminoantipyrine
AAA	Acetylaminoantipyrine
AM	Aminopyrine
B5	Cytochrome b5
BCA	Protein assay reagent
BSA	Bovine serum albumin
BE	Baculovirus-expressed
CL_{int}	Intrinsic clearance
COX	Enzyme cycloxygenase
CYP	Cytochrome P450
DMEs	Drugs metabolizing enzymes
4-DMAA	4-Dimethylaminoantipyrine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
FAA	Formylaminoantipyrine
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
HPLC	High performance liquid chromatography
HLM	Human liver microsomes
IC_{50}	Concentration resulting in 50% inhibition
IS	Internal standard
K_i	Inhibition constant
K_m	Michaelis-Menten constant
4-MAA	4-Methylaminoantipyrine
NADPH	Nicotinamide adenine dinucleotide phosphate
Na	Sodium

NaCl	Sodium chloride
NAT	N-acetyltransferase
NSAIDs	Non steroidal anti-inflammatory drugs
O/R	NADPH-cytochrome P450 reductase
OR/b5	Baculovirus- expressed oxidoreductase with cytochrome b5
PCR	Polymerase chain reaction
PM	Poor metabolizers
Rpm	Rounds per minute
RLM	Rat liver microsomes
RNA	Ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism
Tris	Tris-hydroxymethyl-aminomethane
TD	Tardive dyskinesia
V_{max}	Maximum reaction velocity

1 INTRODUCTION

1.1 Drug metabolism

The majority of drugs undergo a variety of chemical reactions in the liver and, to a much lesser extent, in other organs (e.g., intestinal wall, kidney, lungs). Such reactions include oxidation, reduction, hydrolysis, and conjugation (with glucuronic acid, amino acids, acetate, sulphate, and methyl groups) and are directed towards the production of metabolites that are more ionized, more water-soluble, and less capable of penetrating cell membranes and being sequestrated in tissues. The more polar or water-soluble a compound becomes, the more readily it is excreted through the kidney and hepato-biliary system. This biotransformation is extremely important because most drugs are lipid-soluble weak electrolytes so that they would be readily reabsorbed through the renal tubule or intestine and remain in the body. The rate of metabolism may be influenced by many factors among which the genetic make-up of the individual and drug interactions are the most important. Metabolism of some drugs, the acetylation of isoniazid being the best example, can proceed at a rapid rate in one subgroup of the population and at a slow rate in another genetically defined subgroup of the population. A slow rate may be due to the deficiency of a specific enzyme because of some genetic defect and results in an increased sensitivity to drugs. For example, in subjects with acetyltransferase deficiency, the speed of acetylation and inactivation of isoniazid is decreased and consequently the usual doses of the drug will produce toxic effects.

1.1.1 Specific reactions in drugs metabolism

The specific reactions in drugs metabolism are often divided into Phase-I and Phase-II. Phase-I DMEs, many of which are cytochromes P450, sometimes participate in detoxification of reactive substrates. But they are more often involved in the activation of inert protoxicants, promutagens and procarcinogens to electrophilic intermediates that can bind as adducts to proteins or DNA and/or cause oxidative stress (Dalton et al., 1999; Kidd et al., 1999; Nebert, 2000). Phase-II DMEs (e.g. methyltransferases, UDP glucuronosy-ltransferases, glutathione transferases, sulfo-transferases) are sometimes involved in metabolic activation (Nebert et al., 1996), but they usually conjugate various Phase-I products and other reactive intermediates to form water-soluble derivatives, completing the detoxification cycle.

Therefore, it seems likely, that genetic differences affecting the expression of Phase-I and Phase-II DME might be crucial factors in defining susceptibility to toxicity or cancer caused by drugs and other environmental pollutants. Hundreds of genes coding for drug metabolizing enzymes exist in the human genome. Polymorphism in several such genes causing high levels of one enzyme and low levels of another enzyme in a specific pathway involved in the metabolism of a particular environmental pollutant could lead to 30- or more than 40-fold differences between two individuals in response to that foreign chemical (Nebert, 2000).

Table 1. Phase-I and phase-II DMEs

Enzyme class	Reaction type	Enzymes
	Phase I DMEs	
Oxidation	Hydroxylation, N- and O- dealkylation, desamination, oxidative dehalogenation	Cytochrome P450 monooxygenases
	N- and S-Oxidation	Cytochrome P450 monooxigenases, flavin monooxigenases
	Dehydration	Alcohol dehydrogenases
	Dehydration of amines	Monoamin oxidases
Reduction	Carbonyl reduction	Carbonyl reductases
Hydrolysis	Hydrolysis of epoxides	Epoxide hydrolases
	Hydrolysis of esters	Carboxylesterases
	Hydrolysis of peptides	Peptidases
Others	Oxidation of superoxide anions	Superoxide dismutases
	Peroxidation	Glutathione peroxidases
	Phase- II DMEs	
Conjugation	Glucuronosylation	UDP-glucuronosyltransferases
	Sulfation	Sulfotransferases
	Acetylation	O- and N-acetyltransferases
	Methylation	O-, N- and S-methyltransferases
	Glutathione S-conjugation	Glutathione S-transferases

*Adapted from Elke Störmer, Dissertation, Berlin, 2001.

It is now well recognized that adverse drug reactions may be caused by specific drug-metabolizer phenotypes. This is illustrated by the severe and potentially fatal hematopoietic

toxicity that occurs when thiopurine methyltransferase-deficient patients are treated with a standard dose of azathioprine or mercaptopurine (Krynetski and Evans, 1998).

Another example is the slow acetylator phenotype that has been associated with hydralazine-induced lupus erythematosus isoniazid-induced neuropathies, dye-associated bladder cancer, and sulfonamide induced hypersensitivity reactions. In all cases, acetylation of a parent drug or an active metabolite is an inactivating pathway. *N*-Acetyltransferase is an enzyme that conjugates substrates with a more water- soluble small molecular moiety. Such conjugation reactions are frequently, but not always, detoxifying, in that they often "mask" a more reactive functional group and usually enhance urinary or biliary excretion of substrates. There are many examples in which the combination of a genetic defect in a conjugation pathway (Fig. 1, right) coupled with a wild-type phenotype for an oxidation pathway (Fig.1, left), or other chemical modifications, results in a phenotype particularly pre-disposed to adverse effects from a medication or environmental substance.

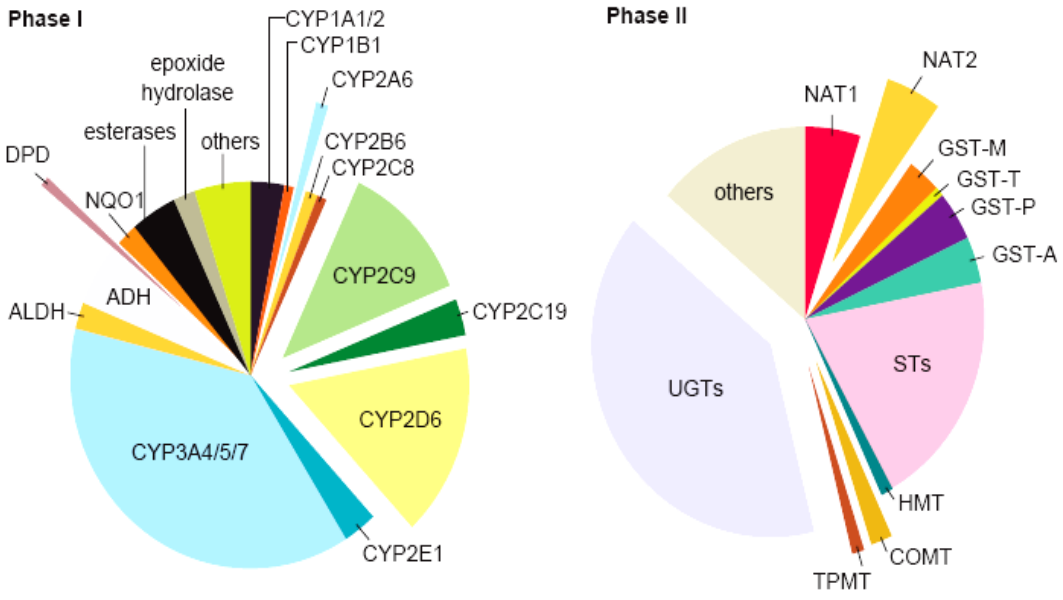

Fig. 1. Participation of specific human liver cytochrome P450 enzymes (left side) and phase-II-enzymes (right side) in drug metabolism. The sizes of the segments refer to the relative number of drugs metabolized by the respective enzyme, e.g. about 40% of all currently used drugs are metabolized by cytochrome P450 3A enzymes (bright blue segment). Figures taken from (Evans and Relling, 1999).

As one example, increased CYP1A activity (an enzyme catalyzing a phase-I oxidation reaction), coupled with slow acetylation (a phase- II conjugation reaction), resulted in less myelosuppression from the active metabolites of the anticancer agent amonafide (Ratain et al., 1996, Evans and Relling, 1999).

Because every individual has a combination of drug- metabolizer phenotypes, given the large number of enzymes involved in drug metabolism, it is apparent that some individuals are destined to have unusual reactions to drugs or to combinations of drugs due to the coincident occurrence of multiple genetic variants in drug- metabolizing enzymes. Such an alignment of genotypes, particularly when coupled with polymorphisms in drug receptors, is likely to constitute part of the mechanism for so-called idiosyncratic drug reactions. In addition to detoxifying and eliminating drugs and metabolites, drug-metabolizing enzymes are often required for activation of prodrugs. Many opioid analgesics are activated by CYP2D6 (Krynetski and Evans, 1998), rendering the 2 to 10 % of the population who are homozygous for non-functional *CYP2D6* mutant alleles relatively resistant to opioid analgesic effects. It is thus not surprising that there is remarkable interindividual variability in the adequacy of pain relief when uniform doses of codeine are widely prescribed. For many genetic polymorphisms of drug-metabolizing enzymes, there is no evident phenotype in the absence of a drug challenge, perhaps because these enzymes are not critical for metabolism of endogenous compounds in physiologically essential pathways. However, some drug- metabolism genotypes may result in a phenotype in the absence of drug; for example, it has been postulated that CYP2D6 poor metabolizers are less pain tolerant than extensive metabolizers because of a defect in synthesizing morphine from codeine (Sindrup et al., 1993b), and that certain forms of dihydropyrimidine dehydrogenase deficiency are associated with mental retardation (Diasio, 1998). Moreover, the risk of some cancers has been linked to polymorphisms in drug-metabolizing enzymes, which may be due to an impaired ability to inactivate exogenous or endogenous mutagenic molecules. As depicted in Fig.1 CYP3A4 is the human enzyme known to be involved in the metabolism of the largest number of medications. Thus far, no completely inactivating mutations have been discovered in the human *CYP3A4* gene, although a common polymorphism in the *CYP3A4* promoter has been recently described (Rebbeck et al., 1998). For enzymes that apparently do not have critical endogenous substrates (for example, *CYP2C19*, *CYP2D6*, and *TPMT*), the molecular mechanisms of inactivation include splice site mutations resulting in exon skipping (for example, *CYP2C19*), gene duplication (for example, CYP2D6), point mutations resulting in early stop codons (for example, *CYP2D6*), amino acid substitutions that alter protein stability or catalytic activity (for example, *TPMT*, *NAT2*, *CYP2D6*, *CYP2C19*, and *CYP2C9*), or complete gene deletions (for example, *GSTM1* and *CYP2D6*). It is remarkable that even for rare phenotypes such as thiopurine methyltransferase deficiency (which occurs in only 1 in 300 individuals), a small number of recurring mutations have been shown to account for most

of the mutant alleles in humans (Krynetski and Evans, 1998). For this and other drug-metabolizing genes, the frequency of SNPs and other genetic defects appears to be more common than the frequency of "1 per 1000 base pairs" that is cited for the human genome. Perhaps it is because some "drug"-metabolizing enzymes are dispensable or redundant with other enzymes (such as CYP2D6 and CYP) that genetic polymorphisms of drug-metabolizing enzymes are so common.

1.2 Cytochrome P450 Enzymes

1.2.1 Discovery and Background

The cytochromes P450 are a superfamily of enzymes which are found in all forms of living organisms. They are responsible for the metabolism of many endogenous compounds, participate in the activation/deactivation of many carcinogens and detoxify many xenobiotics. In particular, in humans they metabolise many drugs and hence are of great interest to pharmacologists and toxicologists. Its is readily identified by a pronounced absorbance band at 450 nm in the soret region of the visible spectrum when the carbon monoxide adduct of the reduced hem protein is formed (Pohl et al., 1984) hence the name P450. Human cytochrome P450 (P450) enzymes catalyze the metabolism of a wide variety of clinically, physiologically, and toxicologically important compounds. The concept of a familial inherited component modulating drug response was described in the 1950s, often in connection with case- reports of unexpected drug response (Hughes et al., 1954; Kalow et al., 1956; Evans et al., 1960). The variation in drug metabolism was ascribed to different metabolic rates in the enzymes either activating or inactivating the drug. The term pharmacogenetics (the study of heritability of drug response) was coined prior to the current knowledge in molecular biology The current explosion of interest for this field stems from technological advance, such as the mapping of the human genome and SNP (single nucleotide polymorphism) maps constituting the basis for our understanding of individual genetic diversity, and the fact that the results of these efforts are publicly accessible. Pharmacogenetics has traditionally focused on polymorphic drug metabolism, even though hereditary differences in drug receptors and drug transportation systems are included in the concept.

The newer term pharmacogenomics comprises all genetic information concerning drug. response, including the large scale systematic search for drug response markers (Bailey et al., 1998).

Genetic polymorphisms in drug- metabolizing enzymes, transporters, receptors, and other drug targets have been linked to inter individual differences in the efficacy and toxicity of many medications. Pharmacogenomic studies are aiming at elucidating the inherited nature of these differences in drug disposition and effects, thereby enhancing drug discovery and providing a stronger scientific basis for optimizing drug therapy on the basis of each patient's constitution. Pharmacogenetics can be separated into two basic components, pharmaco-kinetics (drug metabolism) and pha rmacodynamics (how a drug acts). The cytochrome P450 isoenzymes are a group of heme- containing enzymes found in the liver embedded primarily in the lipid bilayer of the endoplasmic reticulum of hepatocytes, but a remarkable amount is also found in the small intestine. CYP takes part in the metabolism of many drugs, steroids and carcinogens (Guengerich, 1992). And the recognition of the existence of cytochrome P450 hem proteins dates back to the late 1950s, when a carbon-monoxide-binding protein pigment was reported to be present in the endoplasmic reticulum of the liver (Klingenberg, 1958), and to the later identification of the pigment as type cytochrome (Omura and Sato, 1962; Omura and Sato, 1964a; Omura and Sato, 1964b). Shortly thereafter, the ability to serve as terminal oxidase in the metabolism of steroid hormones (Estabrook et al., 1963) and xenobiotics (Cooper et al., 1965) was demonstrated. These observations were quickly followed by recognition that multiple forms of cytochrome P450 exit in the fragments of endoplasmic reticulum, the microsomes (Lu et al., 1971; Guengerich et al., 1982a; Guengerich et al., 1982b).

1.2.2 Function

Cytochromes P450 (P450s) are enzymes involved in the oxidative metabolism of a wide array of endogenous and exogenous molecules including steroids, plant metabolites, prostaglandins, biogenic amines, drugs, and chemical carcinogens. This broad spectrum of reactions is due to multiple P450 isozymes with differing but overlapping substrate specificities. Cytochrome P450 proteins in humans are drug meta-bolizing enzymes and enzymes that are used to synthesize cholesterol, steroids and other important lipids such as prostacyclins and thromboxane A2. These last two are metabolites of arachidonic acid. Mutations in cyto-chrome P450 genes or deficiencies of the enzymes are responsible for several human diseases. Induction of some CYP enzymes is a risk factor for some types of cancers since these enzymes can convert procarcinogens to carcinogens.

P450 enzymes play a major role in drug interactions. Cytochrome P450 enzymes are heme-containing proteins. The heme iron in cyto-chrome P450 is usually in the ferric (Fe^{3+}) state.

When reduced to the ferrous (Fe^{2+}) state, cytochrome P450 can bind ligands such as O_2 and carbon monoxide (CO). The basic reaction catalyzed by cytochrome P450 is monoxygenation in which one atom of oxygen is incorporated into a substrate (RH); the other one is reduced to water with reducing equivalents derived from NADPH. The typical cytochrome P450 catalysed reaction is:

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + R\text{-}OH$$

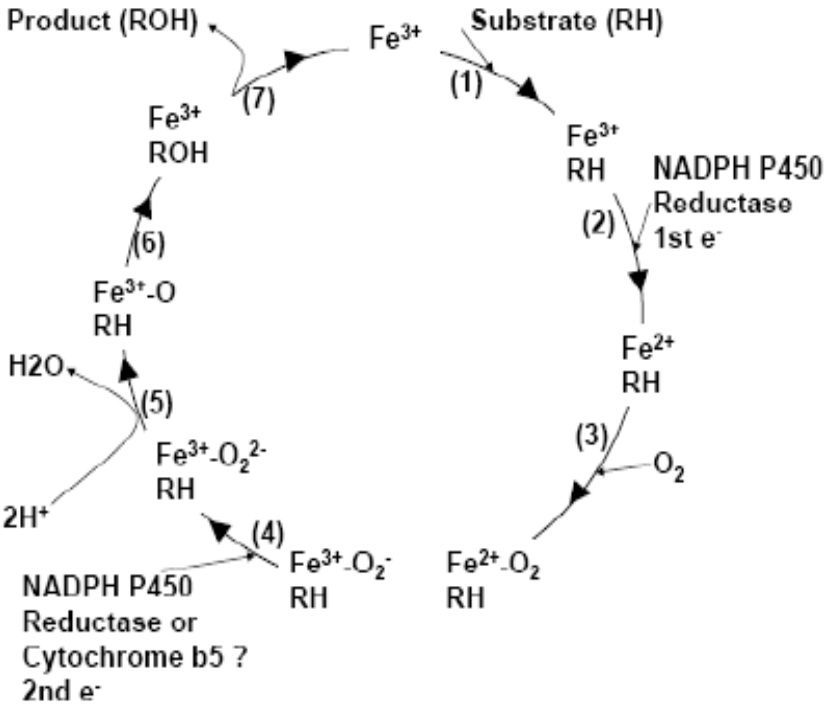

Fig. 2 Catalytic cycle of cytochrome P 450 enzymes (Guengerich and MacDonald, 1990)

The catalytic cycle represented in the above picture may be summarized as follows: (1) The binding of a substrate to a P450 causes a lowering of the redox potential (Ruckpaul et al., 1985), which makes the transfer of an electron favourable from its redox partner, NADH or NADPH. This is accompanied by a change in the spin state of the hem iron at the active site.
(2) The next stage in the cycle is the reduction of the Fe^{3+} ion by an electron transferred from NAD(P)H via an electron transfer chain that depends on a single specific NADPH P450 reductase that contains both FAD and FMN as cofactors (Coon et al., 1975) FAD can accept electrons from NADPH and FMN functions as the single electron carrier.

(3) An O_2 molecule binds rapidly to the Fe^{2+} ion forming Fe^{2+}+ O_2. There is evidence to suggest that this complex then undergoes a slow conversion to a more stable complex Fe^{3+} - O_2 (Archakov et al., 1990). (4) A second reduction is required by the stoichiometry of the reaction. This has been determined to be the rate limiting step of the reaction (Imai et al., 1977). A comparison between the bond energies of O_2, O_2^- , and O_2^{2-} suggest that the Fe^{3+} - O_2^{2-} complex is most favourable starting point for the next stage of the reaction to occur. Some microsomal P450 systems may receive the second electron from NADPH through cytochrome b5; however the mechanism of this interaction still remains speculative (Schenkman and Jansson, 2003). (5) The O_2^{2-} reaction with two protons from the surrounding solvent, breaking the O-O bound, forming water and leaving an (Fe- O) 3+ complex. (6) The Fe- ligated oxygen atom (O) is transferred to the substrate forming an hydroxylated form of the substrate. (7) The product is released from the active site of the enzyme which returns to its initial state.

1.2.3 Evolution

P-450 mixed-function oxidase are enzymes, that catalyze the reduction of oxygen to water. Several studies concerning P450 evolution have been made based on the examination of the phylogenetic tree and its correlation with catalytic activities of cytochrome P450s. The earliest P450s are those that now metabolize steroids and fatty acids. The fatty acid-metabolizing P450 IV family and the steroid-inducible P450 III genes diverged more than 1 billion years ago. The P450 I and P450 II gene families formed about 800 million years ago and these genes are now responsible for the metabolism of drugs and carcinogens (Nelson and Strobel, 1987).

1.2.4 Classification

The classification of the various CYP isozymes employs a three tiered classification based on the conventions of molecular biology: the family (members of the same family display > 40 % homology in their amino acid sequences); subfamily (55 % homology) (Tanaka, 1998) The highest concentration of P450 enzymes involved in xenobiotic bio-transformation is found in the endoplasmatic reticulum (microsomes) of the liver, but P450 enzymes are expressed in almost all tissues.

The human microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families, namely CYP1, CYP2 and CYP3. Liver microsomes also contain P450 enzymes encoded by the CYP4 gene family, the substrates of which include several fatty acids and eicosanoids but relatively few xenobiotics. A classification of all existing 57 human P450s based on substrate class is given in **(**Table 2**)**.

Table 2 Classification of human P450s bassed on major substrate class

CYP Family	Steroids	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1	1A1	1A1	1A1	1A2	1A1	
	1A2	1A2	1B1		1A2	
	1B1	1B1			1B1	
2	2A6	2A6	2A6	2B6	2A6	2A7
	2B6	2A13	2B6	2C8	2B6	2R1
	2C18	2B6	2C19	2C9	2C19	2S1
	2C19	2C8	2D6	2E1	2D6	2U1
	2D6	2C9	2E1	2J2	2E1	2W1
	2E1	2C18	2J2			
	2J2	2C19				
		2D6				
		2E1				
		2F1				
		2J2				
3	3A4	3A4	3A4	3A4	3A4	3A43
	3A5	3A5	3A5		3A5	
	3A7	3A7			3A7	
	3A43					
4	4B1	4B1	4A11	4A11		4A22
		4F12	4B1	4F2		4F11
			4F2	4F3		4F22
			4F3	4F8		4V2
			4F8	4F12		4X1
			4F12			4Z1
5		5A1		5A1		
7	7A1					
	7B1					
8	8A1	8A1		8A1		
	8B1					
11	11A1	11A1			11B1	
	11B1	11B1				
	11B2	11B2				
Others	17	17			19	20
	19	19			24	26C1
	21A2	21A2			26A1	27C1
	27A1	26A1			26B1	
	39A1	51			27A1	
	46A1				27B1	
	51					

(*Adapted from Guengerich FP (Guengerich, 2004))

1.3 Clinical relevance of genetic polymorphisms in drug metabolism

The genetic polymorphisms in drug metabolism and disposition were typically discovered on the basis of phenotypic differences among individuals in the poplation (Mahgoub et al., 1977), but the framework for discovery of pharmacogenetic traits is rapidly changing. Adverse drug reactions are common; they are responsible for a number of debilitating side effects and are a significant cause of death following drug therapy (Lazarou et al., 1998). It is now clear that a significant proportion of these adverse drug reactions, as well as therapeutic failures, are caused by genetic polymorphisms, genetically based interindividual differences in drug absorption, disposition, metabolism, or excretion. Most of the commercially available drugs are metabolized by the phase- I cytochrome P450 superfamily of DMEs. The clinical relevance is best characterized for the genetic polymorphisms in CYP2D6, CYP2C19 and CYP2C9 (Stormer et al., 2000a). CYP2D6 play important roles in the metabolism of beta-blockers, tricyclic antidepressants, antiarrythmic agents, antipsychotic agents and opioids. CYP2C19 is involved in the metabolism of proton-pump inhibitors whereas CYP2C9 metabolizes antidiabetics and anticoagulants. In the recent advances in molecular sequencing technology, gene polymorphisms [such as single- nucleotide polymorphisms (SNPs), and especially SNPs that occur in gene regulatory or coding regions (cSNPs)] may be the initiating discoveries, followed by biochemical and, ultimately, clinical studies to assess whether these genomic polymorphisms have phenotypic consequences in patients. This latter framework may permit the elucidation of polymorphisms in drug metabolizing enzymes that have more subtle, yet clinically important consequences for interindividual variability in drug response. Such polymorphisms may or may not have clear clinical importance for affected medications, depending on the molecular basis of the polymorphism, the expression of other drug-metabolizing enzymes in the patient, the presence of concurrent medications or illnesses, and other polygenic clinical features that impact upon drug response. Almost every gene involved in drug metabolism is subject to common genetic polymorphisms that may contribute to interindividual variability in drug response, are given in (Table 3).

Table 3 Examples of clinically relevant genetic polymorphisms influencing drug metabolism and effects

Gene	Medications	Drug effects linked to polymorphism
CYP2C9 (cytochrome P450 2C9)	Tolbutamide,warfarin, phenytoin, nonsteroidal anti-inflammatoric drugs,	Hypoglycemic effect of oral antidiabetic drugs, anticoagulant effect of warfarin, gastric side effects of nosteroidal anti-inflammatory drugs
CYP2D6 (cytochrome P450 2D6)	Beta-blockers, antidepressants, antipsychotics: codeine, debrisoquin, dextromethorophan, encainide, flecainide, guanoxan, methoxyamphetamine, N-propylajmaline, perhexiline, phenacetin, phenformin, propafenone, sparteine	Cardiac side effects of beta-blockers, Anticholinergic side effects of tricyclic antidepressants, efficacy of antidepressive drugs, tardive dyskinesia from antipsychotics, opioids side effect and efficacy of opioids like codeine and tramadol which are bioactivated by CYP2D6, efficacy and adverse effects (proarrhythmogenic side effects) of antiarrhythmic drugs
DPD (dihydropyrimidine dehydrogenase)	Fluorouracil	Fluorouracil neurotoxicity
TPMT (thiopurine S-methyl-transferase)	6-Mercaptopurine, thioguanine, azathioprine	Thiopurine hematotoxicity and efficacy, risk of secondary cancers
ACE (angiotensin converting enzyme)	Drug targets Enalapril, lisinopril, captopril	Renoprotective effects, cardiac indices, blood pressure immunoglobulin A nephropathy
Potassium channels:		
HERG	Quinidine Cisapride	Drug-induced long QT syndrome Drug-induced torsade de pointes
KvLQT1	Terfenadine, disopyramide, Mefloquine	Drug-induced long QT syndrome
hKCNE2	Clarithromycin	Drug-induced arrhythmia

According to (Evans and Relling, 1999)

1.4 Genetic variability

The frequently studie metabolizing enzymes are the cytochrome P450 (CYP450) isoenzymes, the N-acetyltransferase (NAT) isoenzymes, the UDP glucuro-nosyl transferase, and the methyltransferase. Of these enzymes, the CYP450s are very important because they metabolize drugs into products that are readily excreted into the urine and faeces. In humans, six different forms of CYP P450 (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) are largely responsible for eliminating drugs. The rate of metabolism by several of the cytochrome CYP450 enzyme subfamilies varies, due to genetically determined polymerphisms in all population studied. Recent research using phenotyping and genotyping techniques has reflected the interest and importance of these pharmacogenetic factors in determining drugs responses (Emilien et al., 2000). By inhibiting cytochrome P450, one drug can impair the biotransformation of another drug. Such drug-drug interactions can lead to an excessive pharmacological or toxicological response to the second drug. In this regard, inhibition of cytochrome P450 mimics the effects of a genetic deficiency in P450 enzyme expression. Increased P450 enzyme activity can result from (1) gene duplication leading to overexpression of a P450 enzyme, (2) exposure to environmental factors, such as xenobiotics, that induce the synthesis of cytochrome P450, or (3) stimulation of a pre-existing enzyme by a xenobiotic. By inducing cytochrome P450 one drug can stimulate the metabolism of a second drug and thereby decrease or increase its therapeutic effect. A dramatic effect of this type of drug interaction is the induction of ethinylestradiol metabolism by phenobarbital and rifampin, which can decrease the contraceptive effect of the former drug and lead to pregnancy (Breckenridge et al., 1980). Allelic variants, which arise from point mutations in the wild-type gene, are another source of interindividual variation in P450 activity. Amino acid substitution can increase or, more commonly, decrease P450 enzyme activity, although the effect may be substrate-dependent. Some of the genetic factors that influence P450 activity identified thus far are summarized (Nagata and Yamazoe, 2002), http://www.imm.ki.se/CYPalleles).

The environmental factors which are known to affect P450 expression include medications (e.g. barbiturates, rifampin, isoniazid), food (e.g. cruciferous vegetables, charcoal broiled beef), social habits (e.g. alcohol consumption, cigarette smoking), and disease status (diabetes, inflammation, hyperthyroidism and hypothyroidism). When environmental factors influence P450 enzyme levels, a considerable variation may be observed when xenobiotic biotransformation (e.g. drug metabolism) is measured repeatedly in the same individual.

The variation in therapeutic response to warfarin treatment due to CYP2C9 polymorphism (Furuya et al., 1995; Steward et al., 1997; Aithal et al., 1999) is an example of pharmacokinetics. Another essential aspect of pharmacokinetics is drug absorption. (Hoffmeyer et al., 2000) have demonstrated that polymorphisms in the multidrug- resistence gene-1 influence digoxin absorption. The current use and future perspectives of molecular genetic characterisation of cytochrome P450 enzymes (CYP) for drugs development and drug treatment are summarised. CYP genes are highly polymorphic and the enzymes play a key role in the elimination of the majority of drugs from the human body.

1.4.1 Genetic variability in drug metabolism

In different people and different population, activity of cytochrome P450 enzyme differs. Genetic variation in a population is termed polymorphism when both gene variants exist with a frequency of at least one percent. Such differences in activity may have profound clinical consequences, especially when multiple drugs are given to a patient (Cupp and Tracy, 1998). The explanation for the various polymorphisms are thought to be complex, but perhaps the most interesting is the high expression of CYP2D6 in many persons of Ethiopian and Arabian origin. CYP2D6 is not inducible, so these people have developed a different strategy to cope with the (presumed) high load of toxic alkaloids in their diet- multiple copies of the gene. These CYPs therefore chew up a variety of drugs, making them ineffective - many antidepressants and neuroleptics are important examples. Conversely prodrugs will be extensively activated from example codeine will be turned in vast amounts into morphine. (Ingelman-Sundberg et al., 1999).

1.4.2 CYP2D6 genetic variability

At least 30 drugs, many of them derived from plant alkaloids, have subsequently been shown to be oxidized by CYP2D6, including tricyclic antidepressants, some neuroleptics, beta-blokers, and antiarrhythmic agents such as perhexiline, flecainide, and encainide. Several drugs of abuse including codeine (Chen et al., 1988), hydroquinone (Otton et al., 1993), dextromethorphan (Schmid et al., 1985), and p-methoxyamphetamine (PMA) (Kitchen et al., 1979) are known to be metabolized by this enzyme.

CYP2D6 polymorphisms are clinically important mainly because of the greater likelihood of adverse reactions among persons with poor metabolism, because of the high plasma concentration of the affected drug, and lack of efficacy among persons with ultra rapid metabolism, owing to the consequently low plasma concentration of the affected drug. Because genotyping has not been performed in most people, the response to particular drugs is usually unanticipated. For example, increased cardiovascular toxicity is more likely after usual doses of venlafaxine, a selective serotonin- reuptake inhibitor and adverse effects of tricyclic antidepressants are more frequent in persons with poor metabolism than in those with extensive metabolism. Similarly, persons with poor metabolism have a greater risk of adverse effects when taking metoprolol than do persons with other types of metabolism. The use of typical antipsychotic agents had been observed to induce various adverse effects. One of the most serious effects is tardive dyskinesia (TD). TD is an involuntary movement disorder characterized by a variable combination of orofacial and lingual dyskinesia, tics, grimacing and choreoathetoid hyperkinetic movements in trunk and limbs (Woerner et al., 1991). Several risk factors for TD have been identified. The most important predictors of TD include older age, female gender, organic brain disorders, cigarette smoking, diabetes mellitus and total exposure to antipsychotic drugs (Casey, 1999). Influences of the exposure to typical neuroleptics on TD include the duration of antipsychotic treatment (Casey, 1991; Casey, 1999), and the dosage of antipsychotic medications (Casey, 1991; Casey, 1999). TD occurrence appears to be correlated with higher antipsychotic dosage and consequently, with the plasma level of the drugs (Casey, 1995; Tugg et al., 1997). Therefore, factors that may contribute to a high antipsychotic plasma level might confer the risk of TD. Most typical antipsychotics are in part metabolized by the cytochrome P-450 2D6 (CYP2D6) (Bertilsson et al., 1993; Jerling et al., 1996). Four phenotypes have been identified according to the metabolic activity of CYP2D6: extensive metabolizers, intermediate metabolizers, poor metabolizers (PM) and ultra rapid metabolizers (Sachse et al., 1997; Garcia-Barcelo et al., 2000). The various enzymatic activities and phenotypes of CYP2D6 are encoded by genetic polymorphisms (Andreassen et al., 1997; Armstrong et al., 1997). Several studies had evaluated the relationships between PM genotypes of deficient alleles (*CYP2D6*3*, *CYP2D6*4* and *CYP2D6*5*) on the *CYP2D6* gene and the TD occurrence in a Caucasian population. Nevertheless, their conclusion remained controversial (Armstrong et al., 1997; Ellingrod et al., 2002).

In addition, the incidence rate of the PM phenotype in Chinese or Japanese subjects is lower than in white patients (1% and 5– 10 %, respectively) (Bertilsson et al., 1992) and the deficient *CYP2D6*3* and *CYP2D6*4* alleles, which code the PM phenotype, are rare in Chinese subjects (Garcia-Barcelo et al., 2000). However, early studies showed that the enzymatic activity of CYP2D6 is lower in the Oriental compared with the Caucasian population (Bertilsson et al., 1992). Wang et al. identified a defective allele, *CYP2D6*10*, which resulted from C188T substitution, that may be accounted for the lower CYP2D6 activity in Orientals (Wang et al., 1993). Ohmori et al. (Ohmori et al., 1998) revealed a trend association between *CYP2D6*10* genotypes and TD, and a significant correlation between genotypes and total Abnormal Involuntary Movement Scale (AIMS) scores.

1.4.3 CYP2C19 genetic variability

CYP2C19 is a clinically important enzyme which metabolizes a wide variety of drugs, such as the anti-convulsant mephenytoin (Wilkinson et al., 1989). Anti-ulcer drugs such as omeprazole (Andersson et al., 1992), certain antidepressants (Sindrup et al., 1993a) and (Baumann et al., 1986), the anti malarial proguanil (Ward et al., 1991) and the anxiolytic drugs diazepam . It is also partially responsible for the metabolism of a number of other drugs, such as the β-blocker propranolol metabolism of these drugs in-vivo is polymorphic in humans. Individuals can be characterized as extensive metabolizers or poor metabolizers of drugs metabolized by CYP2C19 in population studies. Poor metabolizers represent 2-5 % of Caucasians, 13- 23 % of Asian populations and as many as 38- 79 % of individuals of some of the islands of Polynesia and Micronesia (Kaneko et al., 1999). African population have been studied less extensively, but the poor metabolizer trait has been reported to be approximately 40% in African- Americans from mid- Tennessee (Edeki et al., 1996), Africans from Zimbabwe and Nigerians (Daniel and Edeki, 1996). Poor metabolizers can experience undesirable side- effects, such as prolonged sedation and unconsciousness after administration of diazepam. This can be a particular problem in Asian patients where the poor metabolizer phenotype is frequent. In contrast, omeprazole has been reported to produce a greater cure rate for gastric ulcers and accompanying Helicobacter pylori infections in CYP2C19 poor metabolizers than in extensive metabolizers because blood levels are higher in these individuals (Goldstein, 2001). For CYP2C19, 16 allelic variants have been identified http://www.imm.ki.se/CYPallele.

CYP2C19*2 and CYP2C19*3 are important, since the CYP2C19*2 allelic variant accounted for 75– 83 % of the poor metabolizer (PM) phenotypes in Caucasian (De Morais et al., 1994) and Asian populations (de Morais et al., 1994). Furthermore, CYP2C19*2 and *3 combined, accounted for 100 % of PM phenotypes in a study of a Japanese population (Ibeanu et al., 1998). CYP2C19*2 variant causes a 681G>A nucleotide change in exon 5 that creates a splicing defect, and CYP2C19*3 is a 636G>A change that generates a stopcodon in exon 4. The distr ibution http://www.imm.ki.se-/CYPallele, (De Morais et al., 1994; de Morais et al., 1994), of these allelic variants within different ethnic groups varies. For instance, Asian populations exhibit higher allelic frequencies of CYP2C19*2 (21– 45%) and CYP2C19*3 (2– 16%) (Roh et al., 1996; Kimura et al., 1998Roh, 1996 #119)) than European-American populations (*2: 13–19%; *3: 0– 0.3%) (Goldstein et al., 1997; Ruas and Lechner, 1997; Xie et al., 1999). By contrast, CYP2C9*2 and CYP2C9*3 appear to be more prevalent in European and American populations (8– 15%; 5– 16%) (Yasar et al., 1999) than in Asian populations (0; 1– 5%) (Sullivan-Klose et al., 1996, Kimura, 1998 #118, Nasu, 1997 #125,{Yoon, 2001 #126). Although the genetic polymorphism of the CYP2C subfamily has been widely studied, there are no data regarding South American populations. Its population is divided ethnically into three main groups: the Amerindian (55 %), Mestizo (30 %) and White (15 %) people. The Amerindian population is represented by approximately 35 ethnic groups, which are clustered based on their linguistic characteristics. The Arawak (mojo), Aymara, Chiquitano, Mataco- Mac'a, Pano, Quechua, Tacana, Tupi-Guarani and Uru-Chipaya are the major ethno- linguistic groups of theAmerindian group. The Mestizo population is the admixture between White and Amerindian populations. This admixture is explained in part by the historical (Aklillu et al., 2003) events of the Spanish Conquest in the Americas that produced a mixture between mostly Quechua and Spaniards. The White population represents a small proportion of the population. In addition to the arrival of the Spaniards during the Spanish Conquest, a significant number of Europeans immigrated to Boliviabe fore and during World War II. http://www.ine.gov.bo.

1.4.4 CYP1A2 genetic variability

CYP1A2, a hepatic enzyme inducible by smoking, metabolizes various chemical procar-cinogens, such as food- derived heterocyclic and aromatic mutagens, *N*- heterocyclics found in tobacco smoke, and difuranocoumarins, to reactive carcinogens (McManus et al., 1990).

It is also involved in the metabolism of several drugs such as paracetamol, theophylline, caffeine, and clozapine (Bertilsson et al., 1994). Endogenous substrates of CYP1A2 include estradiol and uroporphyrinogen. The enzyme has a significant role in chemical carcinogenesis (Eaton et al., 1995) and is induced by its substrates, and a polymorphism in its capacity to activate procarcinogens has been indicated (Minchin et al., 1985). Hepatocellular carcinoma is a common neoplasm, especially in Africa, and is to a great extent caused by the intake of dietary aflatoxin (Uwaifo and Bababunmi, 1984). CYP1A2 has been reported to play a more important role than CYP3A4 in the bioactivation of aflatoxin at low concentrations in human liver microsomes (Gallagher et al., 1996). The antiparasitic drug oltipraz, which is currently on phase- II human clinical trials for its cancer- chemopreventive effect in humans, especially with respect to aflatoxin- associated hepatocarcinogenesis, has been shown to be a potent inhibitor of CYP1A2 (Sofowora et al., 2001). Subjects with higher CYP1A2 activity and exposed to dietary aflatoxin B1 might be at a higher risk for developing hepatocellular carcinoma. Individual differences in CYP1A2 activity may thus influence individual susceptibility to cancer and the therapeutic efficacy of some drugs. The human *CYP1A2* gene, located on chromosome 15, spans about 7.8 kb and contains seven exons. The coding region starts at nucleotide 10 of exon 2 (Ikeya et al., 1989). Exon 2- 6 is highly conserved among human, mouse, and rat. In these species, regions of high conservation have also been found in intron 1 of CYP1A2 (Ikeya et al., 1989) suggesting a possible regulatory role of this intron. Two single-nucleotide polymorphic sites (SNPs), –164G>A and –740T>G, have previously been reported in the intron 1 of human *CYP1A2* gene http://www.imm.ki.se-/CYPalleles-/CYP1a2.htm. The – 164C>A SNP has been suggested to be associated with higher enzyme inducibility by smoking among white persons (Sachse et al., 1997), whereas the – 740T>G has not been functionally characterized. Previous studies on the human *CYP1A2* gene regulation in HepG2 cells have identified two regions of importance for basal expression: a proximal region containing a GC box, a CCAAT box and a TATA box, and a distal region, named "1A2 enhancer," which contains two activator protein-1 sites, a xenobiotic- responsive element, and a hepatic nuclear factor 1 site and a second TATA box) (Quattrochi et al., 1994; Chung and Bresnick, 1995; Chung and Bresnick, 1997). The aryl hydrocarbon receptor null mice show significant decrease in CYP1A2 expression in the liver, suggesting that the xenobiotic-responsive elements may be involved in the regulation of the basal expression (Schmidt et al., 1996).

It is possible that several of these factors, including nuclear factor 1, participate in the tissue-selective activation of *CYP1A2* gene expression and that the absence of any single component may abolish or down- regulate gene expression. The human CYP1A subfamily consists of CYP1A1 and CYP1A2. The former is expressed mainly in extra hepatic tissues, and the latter is almost exclusively expressed in the liver. CYP1A2 is responsible for the oxidative metabolism of drugs such as theophylline, mexiletine, and phenacetin (Distlerath et al., 1985; Sarkar and Jackson, 1994; Nakajima et al., 1998). This enzyme has also been shown to be involved in the metabolic activation of carcinogenic arylamines to produce reactive intermediates (Eaton et al., 1995). Up to 60-fold inter-individual variation in the CYP1A2 activity has been reported (Shimada et al., 1994; Saruwatari et al., 2002). Also, approximately 15- and 40- fold interindividual variations in CYP1A2 mRNA and protein expression levels have been observed in the human liver (Ikeya et al., 1989; Guengerich et al., 1999). These interindividual differences are likely to influence the drug metabolism and to be associated with drug efficacy and safety and cancer susceptibility caused by procarcinogens. Environmental factors have been thought to influence the interindividual differences. Cigarette smoking and intake of oral contraceptive steroids are well established modifiers of CYP1A2 activity (Rasmussen et al., 2002). However, it has been suggested that approximately 35 to 75 % of the interindividual variability in CYP1A2 activity is due to genetic factors (Kendler and Prescott, 1999; Rasmussen et al., 2002). Since *CYP1A2* is inducible by environmental factors, many investigators have tried to identify single nucleotide polymorphisms (SNPs) in the transcriptional regulatory regions: the distal enhancer region, the promoter region, noncoding exon 1, and intron 1. *CYP1A2*1C* (–3860G>A) was reported to be associated with decreased enzyme inducibility in Japanese smokers (Nakajima et al., 1999). *CYP1A2*1F* (–163C>A), located in intron 1, has been suggested to be linked with a higher enzyme inducibility in white smokers (Sachse et al., 1999). Recently, Aklillu et al. reported that the *CYP1A2*1K* haplotype (–739T>G, –729C>T, and –163C>A; all in intron 1) was associated with decreased enzyme activity in Ethiopian non smokers. As for the coding exons, Phe21Leu (*CYP1A2*2*) was first reported from the direct sequencing of DNA from one of eight Chinese subjects (Huang et al., 1999). The other four nonsynonymous SNPs (Asp348Asn, *CYP1A2*3*; Ile386Phe, **4*; Cys406Tyr, **5*; and Arg431Trp, **6*), found in French whites, were reported to alter protein expression levels and/or alter enzymatic activities depending on the substrates (Zhou et al., 2004).

1.5 Investigated known and presumed substrates of cytochrome P450 enzymes

1.5.1 Analgesic-antipyretic drugs

The anti-inflammotory, analgesic and antipyretic drugs are a heterogeneous group of compounds, often chemically unrelated (although most of them are organic acids), which nevertheless share certain therapeutic actions and side effects. The prototypes are aspirin, metamizole and aminopyrine. Hence these compounds are often referred to as aspirin-like drugs they also are frequently called nonsteroidal anti-inflammatory drugs, or NSAIDs, an abbrivation that is used throughout this thesis when refering to these agents. There has been substantial progress in elucidating mechanism of action of NSAIDs. Inhibition of cyclooxygenase (COX), the enzyme responsible for the biosynthesis of the prostaglandins and certain related autacoids, generally is thought to be a major facet of mechanism of NSAIDs. Some of shared properties of NSAIDs are considered first. then the more important drugs are discussed in some detail.

1.5.2 Mechanism of action of NSAIDs

Although NSAIDs had been known to inhibit a wide variety of reactions in-vitro, no convincing relationship could be established with their known anti- inflammatory, antipyretic, and analgesic effects until 1971, when Vane and associates and Smith and Willis demonstrated that low concentration of aspirin and indomethacin inhibited the enzymatic production of prostaglandins. There was, at that time, some evidence that prostaglandins participated in the pathogenesis of inflammation and fever, and this reinforced the hypothesis that inhibition of the biosynthesis of these autacoids could explain a number of the clinical action of the drugs (Higgs and Vane, 1983). Numerous subsequent observations have reinforced this point of view, including the observation that prostaglandins are released whenever cells are damaged, they appear in inflammatory exudates, and NSAIDs inhibit the biosynthesis of prostaglandins in all cells tested. However, NSAIDs generally do not inhibit the formation of eicosanoids such as the leukotrienes, which also contribute to inflammation, nor do they affect the synthesis of numerous other inflammatory mediation. There are differences of opinion as to whether or not NSAIDs may have other actions that contribute to their therapeutic effects.

1.5.3 Metamizole

Metamizole, or dipyrone, chemically [(2,3-dihydro-1,5 dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-yl) me-thylamino] methanesulphonic acid, is an effective and widely used drug in several countries because of its analgesic and antipyretic properties (Agundez et al., 1994). Metamizole was first synthesized by the German company Hoechst AG in 1920, and its mass production started in 1922. Metamizole is a pyrazoline derivative available in oral and parenteral forms acting as inhibitor of cyclooxygenases. It has been used as a nonsteroidal anti-inflammatory agent (Bonkowsky et al., 2002) as well as a potent analgesic and antipyretic drug in many countries for more than 60 years. Oral doses of 0.5 to 1 g (Pereira et al., 1985) are effective in treating fever. Repeated doses (up to 4 times daily) can be administered, the maximum recommended dose is 3 to 4 g daily (Sadusk, 1965). Metamizole is given orally as capsules or at tablets. Sometimes, users of metamizole prefer to swallow the contents of an ampoule for parenteral administration, because they believe that the analgesic effect is quicker than with the usual solid oral forms (Artaza et al., 2002). However, metamizole has been associated with fatal agranulocytosis and was withdrawn from the US market in 1979 (Bonkowsky et al., 2002). The complex metabolism of metamizole has been the subject of many in-vivo studies (Levy et al., 1995). In the pharmacokinetics of metamizole, the specific CYP catalyzing the formation of the primary metabolic step to the active metabolite 4-aminoantipyrine (4-AA) is still not known. The biotransformation pathway of metamizole (Levy, 1986) and (Artaza et al., 2002) is well established. It is nonenzymatically dealkylated in the gastric juice to the active moiety 4-methylaminoantipyrine (4-MAA) (Ergun et al., 2004; Vlahov et al., 1990). 4-MAA undergoes demethylation in the liver to 4-aminoantipyrine (4-AA) (Brune and Otterness, 1988; Flusser et al., 1988). AA undergoes further phase-II biotransformation to acetyl-aminoanipyrine (AAA) by the polymorphic N-acetyltransferase (Fig. 3). Metamizole was developed in times where specific CYP enzymes were not yet known but knowledge of the relevant specific CYP enzymes may help in predicting drug- drug interactions. It may also help in elucidating the relevant bioactivation reactions which in some persons result in agranulocytosis.

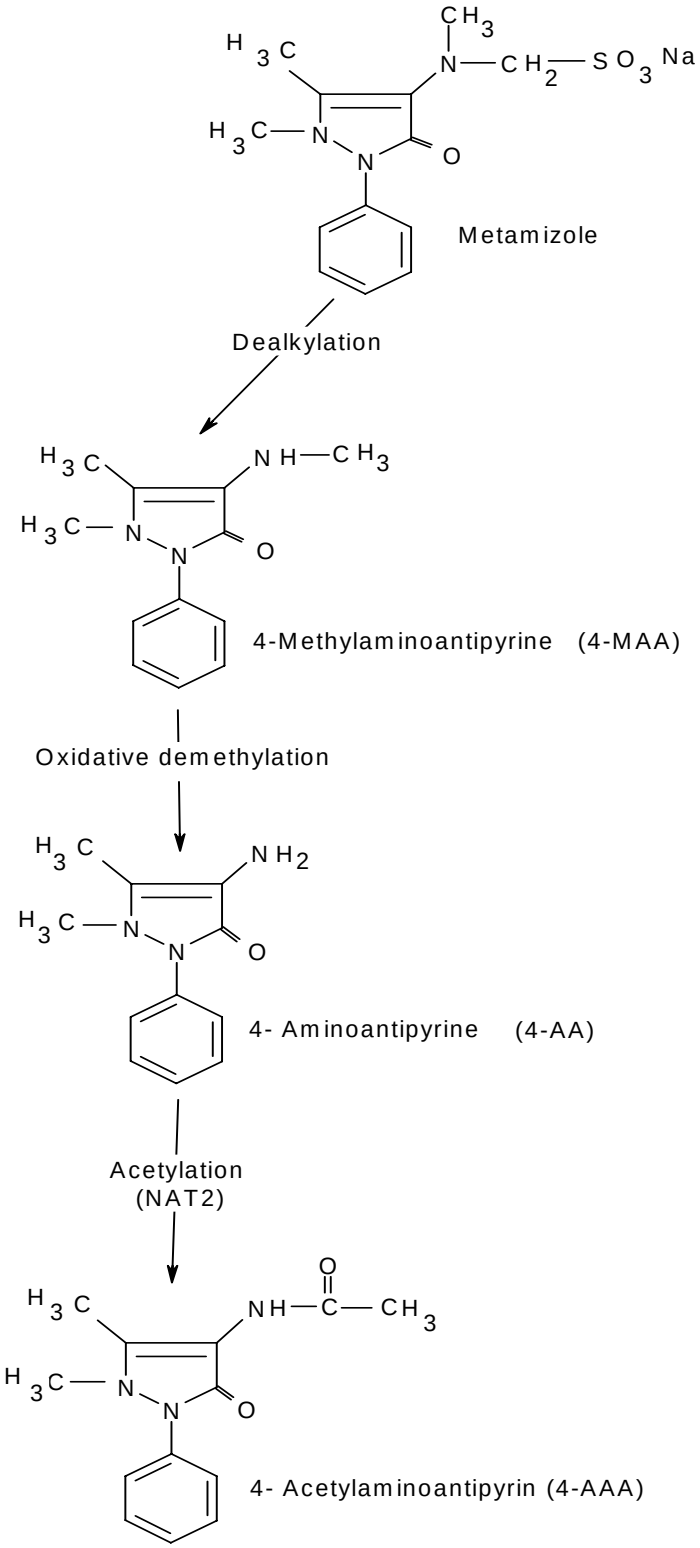

Fig. 3 Structure and biotransformation of metamizole and its main metabolites in man, drawn according to (Geisslinger et al., 1996).

1.5.4 Aminopyrine

Aminopyrine or amidopyrine, chemically 4-(dimethylamino)-1,2 dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one, played a key role in the in-vivo study of human hepatic drug metabolism, because it was widely used as a probe of liver function (Rodzynek et al., 1986; Lane, 1988) and enzymatically active hepatocellular mass (Krahenbuhl et al., 1989; Agundez et al., 1994) in several diseases such as liver cirrhosis (Urbain et al., 1990), chronic hepatitis (Lashner et al., 1988), hepatocarcinoma (Feuer, 1988), or liver ischemia (Metzger and Lauterburg, 1988). Aminopyrine also called amidopyrine, pyrazolone, 4-dimethyl-aminoantipyrine or antipyrine, is a pyrazolone-class analgesic agent used in otic solution (solution for treatment of ear deseases) in combination with other analgesic drugs such as benzocaine, and phenylephrine (Agundez et al., 1995). Aminopyrine is a five-membered lactam ring compound containing two nitrogens and ketone in the same molecule. The lactam structure is an active nucleus regarding pharmacological activity. Aminopyrine may be a useful addition to pharmaceutical ingredients, especially to the class of non-steroidal anti-inflammatory agents used in the treatment of arthritis and other musculoskeletal and joint disorders. Pyrazolone derivatives are also widely used in preparing dyes and pigments has recently been restricted in several countries for human use because of its potential side effects, including mutagenicity (Norkus and Kuenzig, 1985) and agranulocytosis. However, aminopyrine and derivatives, such as dipyrone, are still used in Europe as analgesic drugs. The use of this drug as an indicator for enzymatic activity of the liver after hepatic transplantation has been proposed (Okland et al., 1989). Microsomal cytochrome P450 dependent aminopyrine N-demethylation has been widely studied in rat liver microsomes (Henderson et al., 1986; Imaoka et al., 1988), rabbit intestinal mucosa (Ichihara et al., 1983; Kaku et al., 1985) and rabbit liver (Johnson and Muller-Eberhard, 1977), bovine liver (Tsubaki and Ichikawa, 1985), monkeys (Kastner et al., 1989) and other animal models as well as in humans (Sharonov Iu et al., 1986; Imaoka et al., 1990). The metabolic pathways involved in human disposition of aminopyrine and metamizole have been elucidated, and the involvement of demethylation, formylation and acetylation pathways has been shown in man (Fig. 4) (Brodie et al., 1950; Iguchi et al., 1975; Noda et al., 1976; Volz and Kellner, 1980). The potential side- effects of aminopyrine and metamizole may be related to interindvidual variation in the metabolism of such drugs.

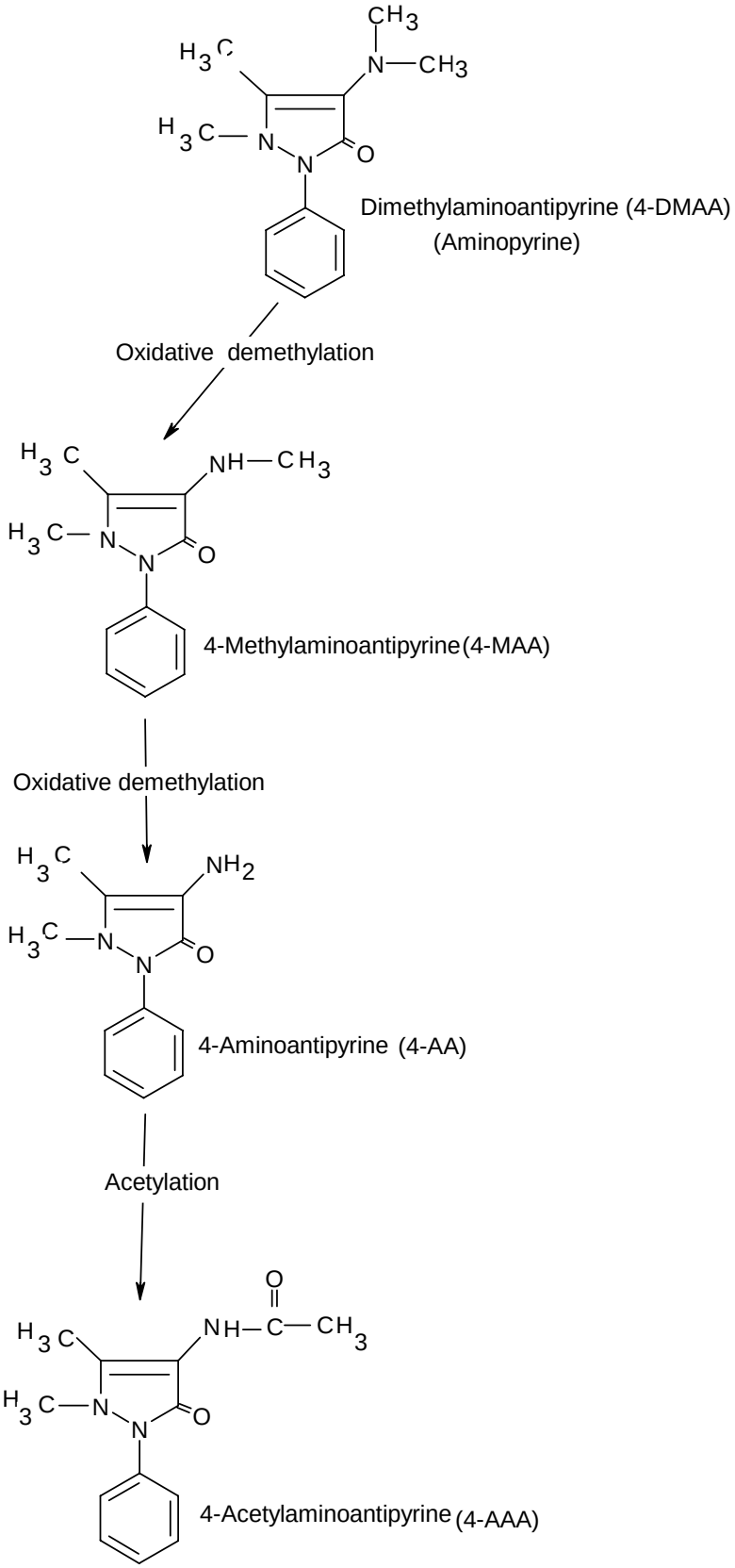

Fig. 4 Structure and biotransformation of aminopyrine and its main metabolites in man, according to (Agundez et al., 1994)

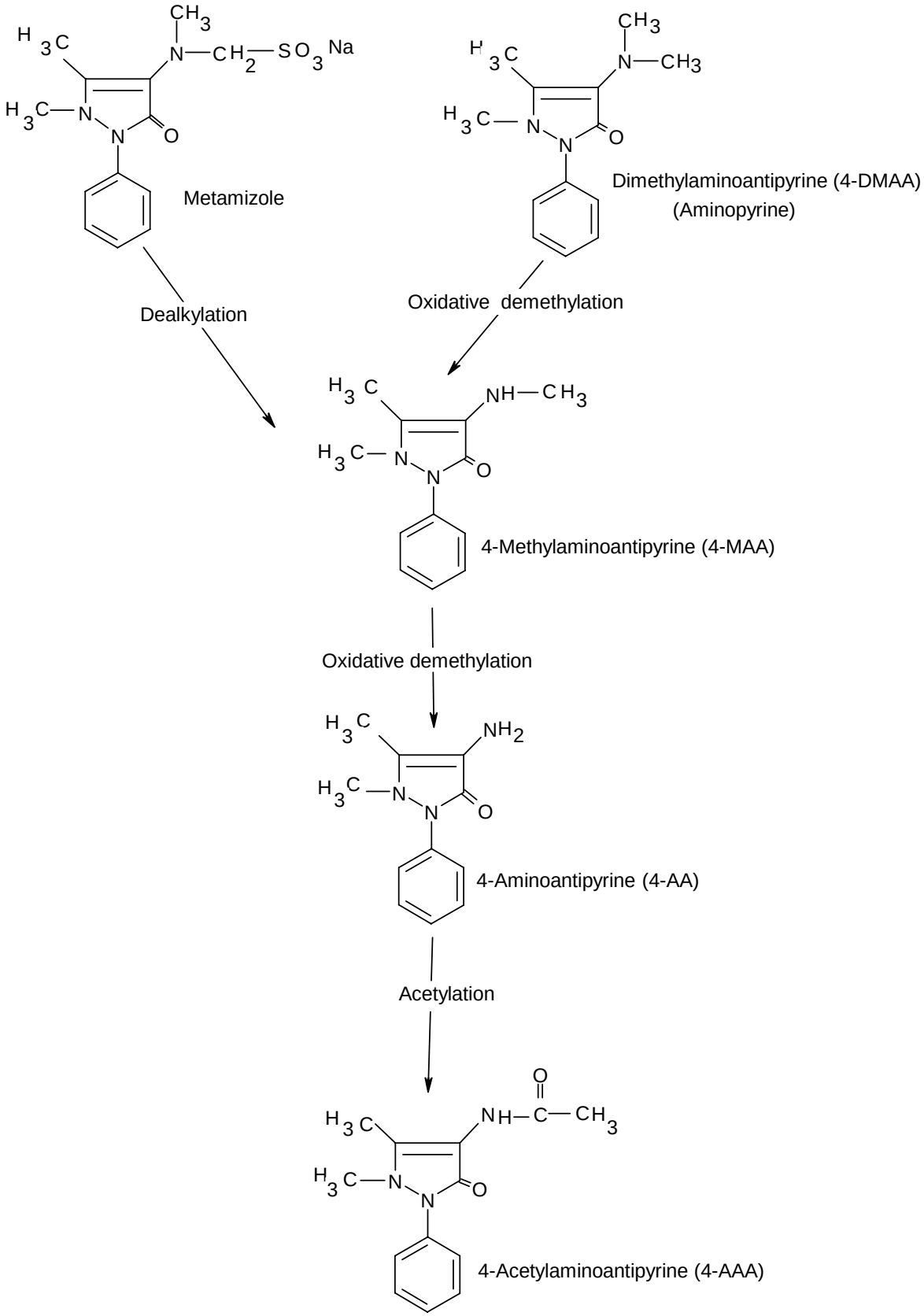

Fig. 5 Structure and biotransformation of aminopyrine and metamizole and their main metabolites in man, according to (Agundez et al., 1994)

2 AIMS OF THE STUDY

The aims of this thesis were to investigate which specific cytochrome P450 enzymes are involved in the biotransformation of the analgesic-antipyretic drugs metamizole and aminopyrine. Such knowledge should help to understand bioactivation mechanisms which may be related to the toxicity of these drugs and such knowledge should also help to understand drug-drug interactions and to understand which genetic polymorphisms might play a role in metamizole or aminopyrine. The analgesic drug metamizole (dipyrone) is non-enzymatically hydrolyzed to 4-methylaminoantipyrine making this the substance of primary interest in our project. In this context, four specific questions should be answered:

1. For the metamizole project, firstly an HPLC assay should be developed and optimized to allow quantification of 4-methylaminoantipyrine and 4-aminoantipyrine in incubation solutions with rat or human liver microsomes or with heterologously expressed specific human cytochrome P450 enzymes. These assays should firstly be optimized based on incubations with rat liver microsomes before going on with human enzymes.

2. Enzyme kinetics of cytochrome P450 mediated metabolism of 4-methyl-aminoantipyrine should then be analyzed with rat liver microsomes, with human liver microsomes from different donors without and with specific inhibitors which should help to identify the relevant enzymes. These results should be confirmed by incubations with heterologously expressed specific human cytochrome P450 enzymes. All data should be analyzed by linearization using Lineweaver and Burk plots and by nonlinear regression analysis.

3. For the aminopyrine project, accordingly an HPLC assay should be developed and optimized to allow quantification of dimethylaminoantipyrine (synonyms: antipyrine, aminopyrine, amidopyrine) and its primary metabolite methylamin-oantipyrine in microsomal incubations.

4. Enzyme kinetics of cytochrome P450 mediated metabolism of dimethyl-aminoantipyrine should then be analyzed with rat liver microsomes, with human liver microsomes from different donors without and with specific inhibitors which should help to identify the relevant enzymes. These results should be confirmed by incubations with heterologously expressed specific human cytochrome P450 enzymes. All data should be analyzed by linearization using Michaelis-Menten equation and by nonlinear regression analysis.

3 MATERIALS AND METHODS

Biotransformation was studied in the subcellular fraction termed microsomes, which is a fraction of membrane vesicles corresponding to the endoplasmic reticulum in the intact cell. Microsomes were isolated from rat and human liver tissues. The impact of genetic polymorphism in one of the enzymes apparently involved in biotransformation of the studied drugs, CYP2C19, was analyzed in the used human liver samples by allelic discrimination. The microsomes were incubated with the substrates metamizole and aminopyrine, respectively. The produced metabolites were identified and quantified using HPLC analysis. Enzyme kinetic data analysis was finally used to determine enzyme kinetic parameters. More specific details about the material and assays used during this investigations are described within this section.

3.1 Materials

3.1.1 Instruments

Instruments	Manufacturer
HPLC pump L-7100	Merck, Darmstadt, Germany
HPLC pump L-600 A	Merck Hitachi, Tokyo, Japan
HPLC autosampler L-7200	Merck, Darmstadt, Germany
HPLC autosampler 655-40 A	Merck Hitachi, Tokyo, Japan
HPLC ultraviolet detector L-7400	Merck, Darmstadt, Germany
HPLC ultraviolet detector 655-A	Merck Hitachi, Tokyo, Japan
Biophotometer (Instrument designed for analysis of DNA, RNA, Oligonucleotide and Proteins)	Eppendorf, Hamburg, Germany
Centrifuge-5810	Eppendorf, Hamburg, Germany
Ultracentrifuge LB-70M	Beckman, Palo Alto, USA
Ultracentrifuge J2-21M/E	Beckman, Palo Alto, USA
Ultracentrifuge T-2190	Centricon, Milano, Italy
Balances	Sartorius AG, Göttingen, Germany
pH Meter	Knick, Berlin, Germany
pH Meter CG 822	Ikamag, Hofheim, Germany
Water bath (for microsomal incubation)	Kötterman, Uetze, Germany

Evaporation thermo-Dux	Techne, Cambridge, UK
Tecan Ultra (Microtiter plate fluorimeter)	Tecan, Crailsheim,Germany
Bio robot (Instrument designed for automating DNA isolation using a solid phase extraction method)	Qiagen, Hilden, Germany
GLF Bi-Dest 2104	Multilab, Düsseldorf, Germany

3.1.2 Consumable materials

Products	Manufacturer
Adhesive PCR film	ABgene, Hamburg, Germany
Adhesive PCR foil seals	ABgene, Hamburg, Germany
Cuvette	Eppendorf, Hamburg, Germany
Tubes 15 ml	Greiner, Flacht, Germany
Tubes 50 ml	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Tubes 1.5 ml with cap	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Tubes 2.0 ml wich cap	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Reaction tubes	Eppendorf, Hamburg, Germany
Nitrile examination gloves	Ansell, München, Germany
Latex gloves	Kimberley-Clark, Koblenz, Germany
Cytostatic gloves	Berner International, Elmshorn, Germany
Pre-column for HPLC	Merck, Darmstadt, Germany
Lichrospher 100 RP-8e column (5 µm particle size, 125 x 4.6 mm inner column dimensions)	Merck, Darmstadt, Germany
Pipettes	Eppendorf, Hamburg, Germany
Pipettes with filter	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Disposable plastic Pipettes	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Centrifuge tubes (20 x 89 mm)	Beckman, Palo-Alto, USA
HPLC caps GL45	Merck, Darmstadt, Germany
HPLC vials	Merck, Darmstadt, Germany
HPLC Lamp	Merck, Darmstadt, Germany
HPLC strips of cap	Merck, Darmatadt,Germany

3.1.3 Chemicals

Chemicals	Manufacturer
4-aminoantipyrine	Sigma chemical, Steinheim, Germany
Aminopyrine	Sigma chemical, Steinheim, Germany
Metamizole	Synopharm, Barsbüttel, Germany
Methylaminoantipyrine	Sigma chemical, Steinheim, Germany
Formylaminoantipyrine	Sigma chemical, Steinheim, Germany
Ketoconazole	Sigma chemical, Steinheim, Germany
Alpha-naphthoflavone	Sigma chemical, Steinheim, Germany
Omeprazole	Sigma chemical, Steinheim, Germany
Moclobemide	Sigma chemical, Steinheim, Germany
Sulphaphenazole	Sigma chemical, Steinheim, Germany
Coumarin	Fluka, Steinheim, Germany
Quinidine	Fluka, Steinheim, Germany
Furafylline	Prof. Dr. Fuhr. University of Cologne, Germany
Fluvoxamine	Synthon, Nigmegen, Netherlands
Tranylcypromine	Röhm Pharma, Weiterstadt, Germany
NADPH	Roche, Mannheim, Germany
NaCl	Appli Chem, Darmstadt, Germany
Protein standard (BSA)	Biorad, Hercules (CA, USA)
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Ampli Tag ^{TM} DNA polymerase	Applied Biosystems, Foster city, USA
Potassium hydroxide pellets	Merck, Darmstadt, Germany
di-sodium hydrogen phosphate	Merck, Darmstadt, Germany
Ortho- phosphoric acid	Merck, Darmstadt, Germany
Sucrose	Merck, Darmstadt, Germany
Pefabloc SC (protease inhibitor, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)	Roche, Mannheim, Germany
Ammonium acetate	Merck, Darmstadt, Germany
Taq DNA polymerase	Qiagen, Hilden, Germany
Taq Man universal PCR master mix	Applied Biosystems, Foster city, USA

Tris base	Applied Chem, Darmstadt, Germany
EDTA	Merck, Darmstadt, Germany
HCl	Merck, Darmstadt, Germany
Bromphenol blue	Roth, Karlsruhe, Germany
Glycerine	Roth, Karlsruhe, Germany

3.1.4 Kits/Reagents

Reagents	Manufacturer
Bradford protein assay	Biorad, München, Germany

3.1.5 Solvents

Solvents	Manufacturer
Acetonitril pro analysis	Merck, Darmstadt, Germany
Methanol pro analysis	Merck, Darmstadt, Germany
Acetonitril pro analysis	J.T. Baker, Deventer, Holland
Methanol pro analysis	J.T. Baker, Deventer, Holland
Double distilled water	Labaratories of the department of Clinical pharmacology, Göttingen University
Ethanol pro analysis	J.T. Baker, Deventer, Holland

3.1.6 Drug metabolizing enzymes

Materials	Type	Manufacturer
Human liver samples	HL001 HL003 HL009 HL010 HL014 HL016	Department of Clinical Pharmacology University, Göttingen, Germany
Rat liver samples	RLM 001 RLM 002	Department of Toxicology, University Göttingen, Germany
Pooled human liver microsomes	HLM	Natutec, Frankfurt am Main, Germany
Recombinant human CYP450 enzymes	CYP1A1 CYP1A2 CYP1B1 CYP2A6 CYP2B6 CYP2E1 CYP2C8 CYP2C9*1 CYP2C19 CYP3A4 CYP3A5 CYP3A7 CYP2D6	Natutec, Frankfurt am Main, Germany

3.2 Methods

3.2.1 In-vitro metabolism

3.2.1.1 Human and rat liver samples

The seven samples of human liver used in the metamizole and aminopyrine study were obtained from a liver bank at the Department of Clinical Pharmacology, Georg-August University in Göttingen, Germany. The donors were white Europeans. The liver samples included nontumorous tissue surrounding primary liver tumors or metastases of various tumors or liver tissues surgically removed for other reasons. The samples of rat liver from male Wistar rats weighing between 180 to 220 g, aged 3 months were kindly provided by the Department of Toxicology at the University of Göttingen.

3.2.1.1.1 Preparation of Human liver microsomes

Human hepatic microsomes (HLM) were prepared by subcellular fractionation as described previously (Pearce et al., 1996). Liver tissue was put into homogenization buffer (20 mM Tris pH 7.4 at 4°C, 5 mM sodium EDTA, 254 mM sucrose, 0.2 mM 4-(2-aminoethyl)-benzolsulfonylfluoride (Pefabloc SC) in an ice bath, liver tissue was weighted, minced with scissors, mixed in a proportion of 1 g tissue in 4 ml buffer, homogenized subsequently with an ultraturax homogenizer at 9,500 rpm for 30 seconds three times, and subsequently homogenized with a Potter-Elvehjem (Glass tube) tissue grinder with 5 to 10 strokes at 1000 rpm.

The obtained homogenate was centrifuged by 3000 g for 30 minutes in an Eppendorf 5810 R centrifuge. The supernatant was removed and centrifuged by 17000 g for 30 minutes at +2°C in a J2-21 M/E Beckman centrifuge. Then the obtained supernatant was removed and centrifuged at 100000 g (29,000 rpm) for 60 minutes in a Beckman Ti50.2 rotor at + 4°C in a L8-70 M Beckman ultracentrifuge. The resulting supernatant (the cytosolic fraction) was removed and stored at -80 °C (not used in the experiments of this project). The resulting pellet (human liver microsomes) was resuspended in microsomal buffer (20 mM Tris, pH 7.4, at 4°C, 5 mM sodium EDTA, 254 mM sucrose and 30% glycerol v/v) and homogenized with a Potter-Elvehjem tissue grider at 1000 rpm. The microsomes were then aliquoted and stored at -80°C until use. The rat liver microsomes were prepared at the same procedures described for human liver microsomes.

3.2.1.1.2 Protein quantification

The protein quantity in microsomal preparations was assessed using the Bradford method (Bradford, 1976) which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic from of the dye, causing a visible color change. The test samples for blank, BSA standards and the protein samples to be tested (unknown samples) were generated. The concentration of protein standards (BSA) ranged from 2 to 20 µg /µl. The dye reagent (1:5 diluted in deionised water) was added to the samples which were then allowed to incubate for 15 min. Thereafter, each sample was measured at 595 nm using a UV-visible spectrophotometer (Bio photometer). The absorbance of BSA standard was plotted as a linear function of its theoretical concentration (Y = m x + b, where Y = absorbance at 595 nm and x = protein concentration) according to the Beer-Lambet law. Using this equation, the protein concentration of each unknown protein samples was calculated based on their measured absorbance.

3.2.2 In-vitro incubation

3.2.2.1 Metamizole

The in-vitro microsomal incubation of metamizole was conducted as described in the following experimental protocol (Table 4). Precisely, stock solutions of 4-methyl-aminoantipyrine ranging from 0.5 to 1.0 mM were prepared in methanol. Volume of these stock solutions representing one of six concentration levels (25, 50, 100, 400, 800 and 1200 µmo/l) were evaporated under a stream of nitrogen for 10 min. The residue was then redissolved in 50 µl of 50 mM potassium phosphate buffer (pH 7.4). Recombinant human CYP450 enzymes, HLM or RLM pre-diluted in the same phosphate buffer were added in a volume 25 µl following a previously described method (Evert et al., 1997). The reaction (total volume 100 µl) was initiated by the addition of 25 µl of 10 mM NADPH, dissolved freshly in the same incubation buffer, and allowed to proceed for 10 to 20 min at 37°C in opened Eppendorf tubes in a shaking water bath. In some experiments, the incubation mixture was adjusted to a final volume 200 µl.

The incubations consisted of (final concentration given) 50 mM potassium phosphate buffer (pH 7.4), BE cytochrome P450 (0.4 - 0.6 pmol/ µl), HLM and RLM (5 - 10 mg protein /ml), 2 mM NADPH and 25 to 1200 µmol/l 4-methylaminoantipyrine in a final volume of 100 µl. The reactions were stopped by adding ice-cold acetonitrile (100 µl). The resulting mixture was centrifuged at 13000 rpm for 5 min. 100 µl of the supernatant were used for HPLC analysis. The formation of 4-aminoantipyrine was linear with time between 6 and 10 min.

Table 4 In-vitro microsomal incubation (example of an experimental protocol)
Analysis of 4-methylaminoantipyrine metabolism by HLM without inhibitors

4-methylaminoantipyrine final concentration in 100 µl incubation volume						
	25 µM	50 µM	100 µM	400 µM	800 µM	1200 µM
4-MAA 0.5 mM in methanol (µl)	10	20	40			
4-MAA 1.0 mM in methanol (µl)				80	160	240
Methanol (µl)	230	220	200	160	80	0
Evaporation / N_2 37 °C (min)	10	10	10	10	10	10
Addition of phosphate buffer (µl)	50	50	50	50	50	50
Pre- incubation (min)	5	5	5	5	5	5
Addition of human liver microsomal solution 10 mg /ml phosphate buffer (µl)	25	25	25	25	25	25
Pre-incubation (min)	10	10	10	10	10	10
Addition of 10 mM NADPH (µl)	25	25	25	25	25	25
Incubation in opened Eppendorf Tubes (min)	20	20	20	20	20	20
Addition of ice-cold acetonitrile (µl)	100	100	100	100	100	100
Centrifugation at 14000 rpm (min)	5	5	5	5	5	5

3.2.2.1.1 Determination of inhibition characteristics.

The 4-methylaminoantipyrine was incubated for 20 min together with the selective chemical inhibitors ketoconazole (CYP3A4), alpha-naphthoflavone (CYP1A1), coumarin (CYP2A6), omeprazole (CYP2C19), quinidine (CYP2D6), tranylcypromine (CYP2C19), fluvoxamine (CYP1A2 and CYP2C19), furafylline (CYP1A2), moclobemide (CYP2C19) and (CYP2D6) and sulphaphenazole (CYP2C9). The inhibitors were dissolved in methanol or water. The concentrations of the inhibitors were 50, 100, 500 µM. Aminopyrine was incubated together with selective chemical inhibitors moclobemide, coumarin, ketoconazole, alpha-naphthoflavone, furafylline and quinidine at the same procedures described for 4-methylaminoantipyrine.

Table 5 Experimental protocol of measuring methylaminoantipyrine metabolism by HLM with inhibitors

4-methylaminoantipyrine final concentration in 100 µl incubation volume						
	25 µM	50 µM	100 µM	400 µM	800 µM	1200 µM
4-MAA 0.5 mM in methanol (µl)	10	20	40			
4-MAA 1.0 mM in methanol (µl)				80	160	240
Addition of inhibitor (µl) solution*	50	50	50	50	50	50
Methanol (µl)	230	220	200	160	80	0
Evaporation / N_2 37 °C (min)	15	15	15	15	15	15
Addition of phosphate buffer (µl)	50	50	50	50	50	50
Pre-incubation (min)	5	5	5	5	5	5
Addition of human liver microsomal solution 10 mg /ml phos phate buffer (µl)	25	25	25	25	25	25
Pre-incubation (min)	10	10	10	10	10	10
Addition of 10 mM NADPH (µl)	25	25	25	25	25	25
Incubation in opened Eppendorf Tubes (min)	20	20	20	20	20	20
Addition of ice cold acetonitrile (µl)	100	100	100	100	100	100
Centrifugation at 14000 rpm (min)	5	5	5	5	5	5

*The inhibitors were prepared as two solutions: solution1 with 100 µ M of the inhibitors and solution 2 with 1 mM of the inhibitors. For 50 µM final inhibitor concentration, 50µl from solution 1, for 100 µM a 100 µl from solution 1 and for 500 µM a 50 µl from solution 2 were added.

3.2.2.1.2 Incubations with heterologously expressed isolated human CYP450s

The recombinant human CYP450 enzymes, pre-diluted in the 50 mM potassium phosphate buffer (pH 7.4), were added in a volume of 25 µl following a previously described method (Evert et al., 1997). The reaction (total volume 100 µl) was initiated by the addition of 25 µl of 10 mM NADPH, dissolved freshly in the same incubation buffer, and allowed to proceed for 20 min at 37°C in opened Eppendorf tubes in a shaking water bath as shown in Table 6.

Table 6 Experimental protocol of the incubations of methylaminoantipyrine with heterologously expressed CYP450 enzymes

4-methylaminoantipyrine final concentration in 100 µl incubation volume						
	25 µM	50 µM	100 µM	400 µM	800 µM	1200 µM
4-MAA 0.5 mM in methanol (µl)	10	20	40			
4-MAA 1.0 mM in methanol (µl)				80	160	240
Methanol (µl)	230	220	200	160	80	0
Evaporation / N_2 37 °C (min)	10	10	10	10	10	10
Addition of phosphate buffer (µl)	50	50	50	50	50	50
Pre- incubation (min)	5	5	5	5	5	5
Addition of CYP450 solution 0.6 pmol/µl in phosphate buffer (µl)	25	25	25	25	25	25
Pre-incubation (min)	10	10	10	10	10	10
Addition of 10 mM NADPH (µl)	25	25	25	25	25	25
Incubation in opened Eppendorf Tubes (min)	20	20	20	20	20	20
Addition of ice cold acetonitrile (µl)	100	100	100	100	100	100
Centrifugation at 14000 rpm (min)	5	5	5	5	5	5

3.2.2.2 Aminopyrine

Stock solutions of aminopyrine (ranging from 0.5 to 1.0 µM) were prepared in methanol and were allowed to evaporate under N_2 as in the following experimental protocol (Table 7). Aminopyrine was then dissolved in 50 µl of 50 mM potassium phosphate buffer (pH 7.4). The final incubation volume was 100 µL. The end concentration of aminopyrine ranged from 25 to 1200 µM. The recombinant cytochrome P450 enzymes or the HLM were added to the reaction mixture in a volume of 25 µl prior to the start of the reaction with NADPH, following a previously described method (Evert et al., 1997) and allowed to proceed for 20 min at 37°C in opened Eppendorf tubes in a shaking water bath, and stopped by the addition of 100 µl ice-cold methanol containing 25 µM sulphaphenazole as an internal standard. The incubation mixtures were then centrifuged at 10000 g for 5 min. Supernatants were transferred into new tubes and 100 µl were used for the HPLC analysis. The formation 4-methylaminoantipyrine was linear with time between 4 and 10 min and with protein ranging from 10 mg/ml for HLM and from 0.6 pmol for baculovirus-expressed cytochromes. The substrate consumption was <10 % over the incubation time (20 min).

3.2.2.2.1 Determination of inhibition characteristics

The 4-dimethylaminoantipyrine was incubated for 20 min together with the selective chemical inhibitors ketoconazole (CYP3A4), alpha-naphthoflavone (CYP1A1), coumarin (CYP2A6), quinidine (CYP2D6), furafylline (CYP1A2), and moclobemide (CYP2C19 and CYP2D6). The inhibitors were dissolved in methanol or water. The concentrations of the inhibitors were 50, 100, 500 µmol/l as described in the above experimental at the procedures described for methylaminoantipyrine (Table 5).

Table 7 In-vitro microsomal incubations (example of experimental protocol) of dimethylaminoantipyrine with HLM.

4-Dimethylaminoantipyrine final concentration in 100 µl incubation volume						
	25 µM	50 µM	100 µM	400 µM	800 µM	1200 µM
4-DMAA 0.5 mM in methanol (µl)	10	20	40			
4-DMAA 1.0 mM in methanol (µl)				80	160	240
Methanol (µl)	230	220	200	160	80	0
Evaporation / N_2 37 °C (min)	10	10	10	10	10	10
Addition of phosphate buffer (µl)	50	50	50	50	50	50
Pre-incubation (min)	5	5	5	5	5	5
Addition of human liver microsomal solution 10 mg /ml phosphate buffer (µl)	25	25	25	25	25	25
Pre-incubation (min)	10	10	10	10	10	10
Addition of 10 mM NADPH (µl)	25	25	25	25	25	25
Incubation in opened Eppendorf tubes (min)	20	20	20	20	20	20
Addition of ice cold acetonitrile (µl)	100	100	100	100	100	100
Centrifugation at 14000 rpm (min)	5	5	5	5	5	5

The in-vitro incubations with chemical inhibitors and with heterologously expressed isolated cytochrome P450 enzymes was conducted as described in the table above and as described for metamizole.

3.2.3 HPLC analysis and chromatographic conditions

3.2.3.1 Metamizole

HPLC for analysis of metamizole and metamizole metabolites was performed similar as described earlier (Asmardi and Jamali, 1983; Geisslinger et al., 1996). The incubation mixtures were centrifuged at 14000 rpm for 5 min. Supernatants were transferred into new tubes and 100 µl used for HPLC analysis. The HPLC system consisted of a L-600A pump (Merck, Hitachi Tokyo, Japan) and 655A-40 autosampler (Merck, Hitachi Tokyo, Japan). The system was equipped with a LiChrospher 100 (Å pore size) RP-8e select column with 5 µm particle size (Merck, Darmstadt, Germany) with internal dimensions of 4 mm x 125 mm preceded by a pre-column (100 Å, diol coated, 5 µM particle size) and some experiments were on a HPLC system consisting of a L-7100 pump (Merck, Darmstadt, Germany) and L-7200 autosampler (Merck, Darmstadt, Germany). The system was equipped with a LiChrospher 100 RP-8e select column 5 µm particle size (Merck, Darmstadt, Germany) preceded by a pre-column (100 Diol, 5 µM). The mobile phase consisted of 75 % (v/v) of 50 mM sodium phosphate buffer (pH 6.0) and 25% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with an ultraviolet (UV) detector (655 A Merck Hitachi Tokyo, Japan) linked to computer data system. The injection volume in these analyses was 40 µl, and the retention times of 4-methylaminoantipyrine (4-MAA), 4-aminoantipyrine (4-AA), and internal standard 4-dimethylaminoantipyrine (4-DMAA) were 10.30, 7.70 and 16.75 minutes, respectively.

3.2.3.2 Aminopyrine

The HPLC system consisted of a L-600A pump (Merck, Hitachi Tokyo, Japan) and 655A- 40 auto sampler (Merck, Hitachi Tokyo, Japan). The system was equipped with LiChrospher 100 RP-8e select column (5 µm particle size, 100 Å pore size, 4 x 125 mm internal dimensions; Merck, Darmstadt, Germany) preceded by a pre-column (100 Diol, 5 µM). The mobile phase consisted of 80 % (v/v) of 50 mM sodium phosphate buffer (pH 6.0), acetonitrile 19 % (v/v) and 1 % (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm, linked to computer data system with an ultraviolet (UV) detector (655 A Merck Hitachi Tokyo, Japan). The injection volume in these analyses was 40 µl, and the retention times of 4-methylaminoantipyrine (4-MAA), 4-dimethylamino-antipyrine (4-DMAA), and internal standard sulphaphenazole were 6.00, 7.70 and 9.60 minutes, respectively.

3.3 Data analysis

3.3.1 Software

Table 8 Computer programs applied

Software	Application	Source
HPLC integration software AIDA software	Signal quantification	Merck, Darmstadt, Germany
Excel 2002 (Microsoft Office)	Linear regression analysis and other statistical calculations	Microsoft, Redmont, WA, USA
Sigma Plot S/PW 9.0	Nonlinear regression Michaelis-Menten enzyme kinetic analysis (K_m V_{max})	Chicago, IL, USA
Regress 3. EXE	Linear regression	Dr. Meineke, Clinical Pharmacology University, Göttingen, Germany

Table 9 Electronic Databases used in this dissertation

Database	URL
Directory of P450- Containing Systems	http:// WWW.icgeb.trieste.it
Drugs Metabolized by Cytochrome P450	http://WWW.georgetown.edu/departments/pharmacology/clinlist.html
Pubmed (NIH)	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
Cytochrome P450 Allele Nomenclature	http://www.cypalleles.ki.se

3.3.1.1 Calculations and estimation of enzyme kinetic parameters

3.3.1.2 Metamizole

3.3.1.2.1 Calculation of metamizole concentrations from the HPLC chromatograms

Concentrations of 4-MAA formation in the incubation samples were calculated by the aid of calibration samples (calibration range: 1 – 10 µg/ml). Peaks of the substances to be measured (S) were electronically integrated and the ratio of the Peak area of S over the peak area of the internal standard (4-dimethylaminoantipyrine) was plotted against the standard concentrations. Regession analyses for these calibrations were performed with the programm regress3.exe.

Table 10 Table with calibration data from one series of AA analysis.

Concentration µg/ml	Peak area 4-MAA	Peak area 4-AA	Peak area IS	4-MAA µg/ml	4-AA µg/ml
1	105	107	2461	1.00	1.00
2	392	384	2467	1.96	1.96
5	1021	1034	2524	4.98	5.15
10	1982	1861	2467	10.12	9.83

3.3.1.2.2 Calculation of enzyme kinetic constants V_{max} and K_M

From the HPLC-quantified 4-AA the formation rate (pmol / mg / min) was calculated The formation rates corresponding to the six substrate concentrations (see incubation conditions above) were then subjected to a regression analysis in order to estimate K_M and V_{max} according to the Michaelis-Menten equation by nonlinear regression analysis with the programm SigmaPlot.

3.3.1.2.3 Determination of the IC_{50} and K_i for the Inhibition

The K_i was determinated by Sigma plot 9.0 and the IC_{50} were determinated with 50 µM of 4-methylaminantipyrine incubated at 37 °C for 20 min with different concentration of inhibitors. The IC_{50} values were then calculated with the equation of (Naritomi et al., 2003).

3.3.1.3 Aminopyrine

3.3.1.3.1 Calculation of 4-DMAA concentrations from the HPLC analyses

Concentrations of 4-DMAA formation in the incubation samples were calculated by the aid of calibration samples (calibration range: 1 – 10 µg/ml). Peaks of the substances to be measured (S) were electronically integrated and the ratio of the Peak area of S over the peak area of the internal standard (sulphaphenazole) was plotted against the standard concentrations. Regession analyses for these calibrations were performed with the programm regress3.exe.

Table 11 Table with calibration data from one series of AA analysis.

Concentration µg/ml	Peak area MAA	Peak area 4-4-DMAA	Peak area IS	MAA µg/ml	DMAA µg/ml
1	105	60	1653	1.00	1.04
2	392	142	1587	1.96	1.81
5	1021	922	1592	4.98	5.12
10	1982	2037	1620	10.12	10.31

3.3.1.3.2 Calculation of enzyme kinetic constants V_{max} and K_M

From the quantified 4-MAA the formation rate (pmol/ mg protein / min) was obtained. The formation rates corresponding to the six substrate concentrations were then subjected to a

regression analysis in order to estimate K_M and V_{max} according to the Michaelis-Menten equation. The Michaelis-Menten equation is given in the following formula where V representes the velocity of product formation and [S] the respective substrate concentrations:

$$V = \frac{V_{\max} \cdot [S]}{K_M + [S]}$$

3.3.2.2.3 IC_{50} and K_i for the Inhibition

These constants were analysed for aminopyrine and for metamizole in the same manner. For analysis of the inhibition constant (K_i) and the concentration of the inhibitor resulting in 50% inhibition (IC_{50}), the Cheng and Prusoff equation was used as follows:

$$K_i = \frac{IC_{50}}{\frac{[Substrate]}{K_M} + 1.0}$$

3.3.2 Predication of pharmacokinetic clearance

The enzyme kinetic parameters of recombinant human CYP450 enzymes and human liver microsomal were used to estimate the in-vitro intrinsic clearance for the reaction from 4-methylaminoantipyrine to 4-aminoantipyrine and for the reaction from 4-dimethylaminoantipyrine to 4-methylaminoantipyrine. The intrinsic clearance was calculated at $CL_{int} = V_{max} / K_m$ (Houston, 1994; Naritomi et al., 2003).

3.4 Method validation

3.4.1 Incubation

3.4.1.1 Solubility

All investigated substances were dissolved in the buffer under the concentration ranges used. Some chemical inhibitors were dissolved in methanol. Control experiments detected >92 % of the substrates (4-methylaminoantipyrine and 4-dimethylaminoantipyrine) in the buffer following incubation at 37 °C.

3.4.1.2 Standard curves

Standard curves were obtained by injecting the extracts of microsomes from one liver without NADPH generating system and with NADPH but zero incubation time, standard peak were obtained by injecting 1, 2, 5, 10 µg /ml from reference substrates and peak area ratios relative to the internal standard were plotted verus concentrations of the standard.

3.4.2 HPLC analysis

3.4.2.1 Limit of detection (limit of quantification)

The limit of detection was 0.25, 0.031, and 0.015 µg/ml of 4-dimethylaminoantipyrine, 4-methyl-aminoantipyrine and 4-aminoantipyrine respectively and the limit of quantification was 1 µg/ml for all three substances. The retention time of chemical inhibitors (to document exclusion of interference with the analysis of 4-methylaminoantipyrine and 4-dimethylaminoantipyrine was 15.20 for furafylline. The other chemical inhibitors did not show any signal within the range of retention time considered for this analysis.

3.4.2.2 Intra-day variability (inter-day variability)

The intra-assay coefficient (inter- assay coefficient) of variation was determined by measuring the formation of the produced targeted metabolite in a series of n (ranging from 15 to 20) identical incubation on the same day (different days) using aliquots of the same HLM lot. The intra-assay coefficient of variation for the formation of 4-aminoantipyrine was 5.5%. The inter- day coefficient of variation for the formation of 4-aminoantipyrine was 6.5%. The intra-assay coefficient of variation for the formation of 4-methylaminoantipyrine was 5.5 %. The inter-day coefficient of variation for the formation of 4-methylaminoantipyrine (4-MAA) was 13.8 %.

4 RESULTS

4.1 Investigations of the metabolism of metamizole by RLM

HPLC Analysis. The substances 4-aminoantipyrine (4-AA) (peak 1, Fig. 6) and 4-methylaminoantipyrine (4-MAA) (peak 2) have been measured with HPLC as illustrated in a chromatogram obtained from injection of 20 µl of standard solution with 25 µmol/l of 4-AA and 4-MAA, each. As shown in the figure, the separation was completed within 10 min. The retention times were 5.08 min for 4-AA and 6.72 min for 4-MAA (Fig. 6).

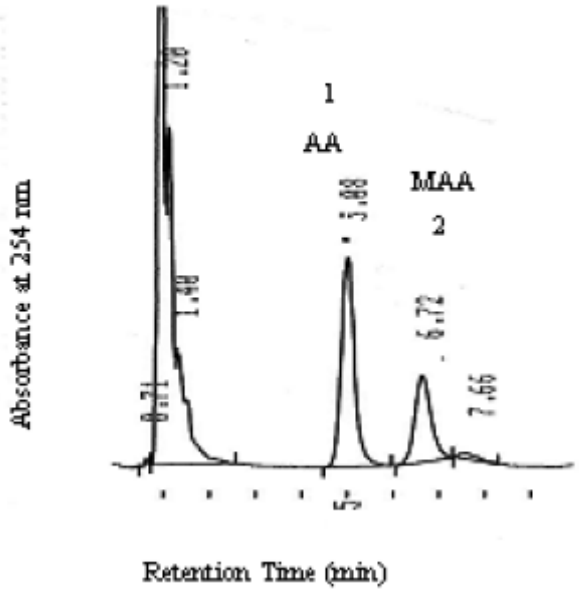

Fig. 6 Typical chromatogram of the main metabolites of metamizole obtained from standard samples of reference substance. Peaks: AA (5.08 min), MAA (6.72 min). The mobile phase consisted of 80 % of 50 mM sodium phosphate buffer (pH 6. 0), 15% acetonitrile and 5% methanol degassed before use, and the flow rate was 1. 0 ml/min. Stationary phase was a reversed phase (RP) octylsilane endcapped (5 µm particle size, 125 x 4 mm internal dimensions) column equipped with a pre-column (diol-coated, 5 µm particle size).

Incubation of 4-methylaminoantipyrine with all microsomal preparations resulted in the formation of the 4-aminoantipyrine as the only detectable metabolite of 4-methyl-amino-antipyrine as shown in Fig. 7.

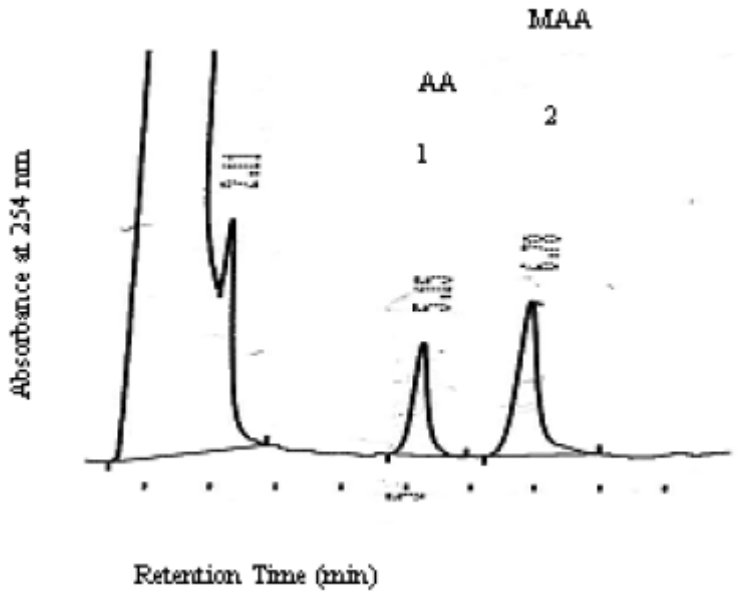

Fig. 7 Elution profiles of 4-methylaminoantipyrine and its metabolites by HPLC with a RP-8 endcapped (5 µm particle size, 125 x 4 mm internal dimensions) column equipped with a pre-column (100 Å pore size, diol-coated, 5 µm particle size). The mobile phase consisted of 80 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0), 15% acetonitrile and 5% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with a detector linked to a computer data system. The chromatogram shows 4-methylaminoantipyrine and its metabolite formed by rat liver microsomes. A reaction mixture (200 µl) with 1.25 mg/ml (final concentration) of microsomal protein from male Wistar rats, 1.0 mg/ml of NADPH and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC. Peaks: 4-AA at 5.15 min and 4-MAA at 6.78 min.

Two different preparations of rat liver microsomes were used. Mean V_{max} (arithmetic mean of 5 incubations) was 201 pmol/min (standard deviation, SD, 42.2 pmol/min) and mean K_M was 20.9 µmol/l (SD 3.8) as shown in Fig. 8. The corresponding intrinsic clearance was 9.61 µl/min (SD 3.9). The quantity of microsomal protein was 0.250 mg protein in each assay. HPLC chromatograms from incubations of the metamizole metabolite 4-methylaminoantipyrine with hepatic microsomes of untreated male Wistar rats weighing between 180 to 220 g, aged 3 months and two control incubation samples without NADPH and zero incubation time are shown in (Fig. 9) right and left respectively.

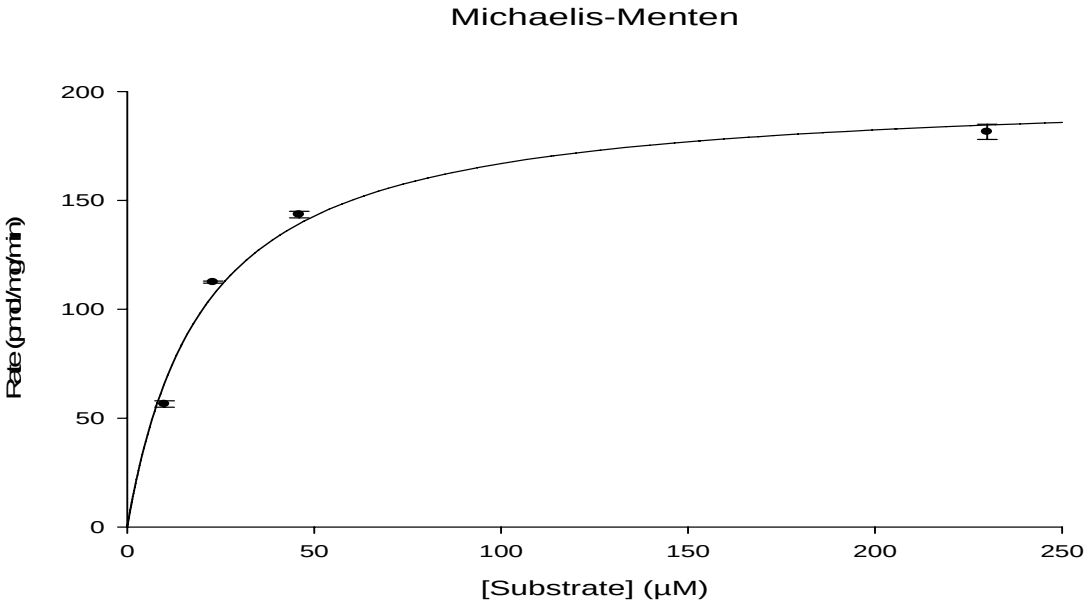

Fig. 8 Kinetic plot of demethylation of 4-methylaminoantipyrine by RLM. The reaction mixture (200 µl) included 1.25 mg/ml (final concentration) of microsomal protein of male Wistar rats and 1.0 mg/ml of NADPH. 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC.

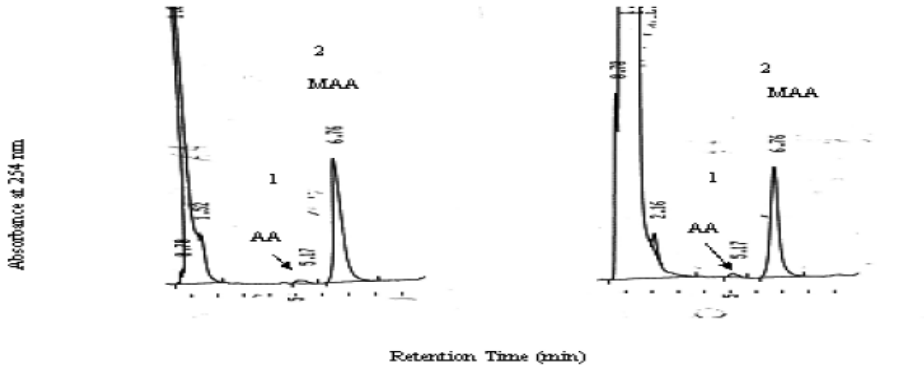

Fig. 9 (Right) Chromatogram of an extract from rat liver microsomes obtained after 20 min incubation of MAA without NADPH. Peaks: MAA at 6.76 min. (Left) Extract from rat liver microsomes obtained 0 min after incubation with MAA. The metabolite was not seen in the right and left by HPLC with a RP-8 endcapped (5 µm partical size; 125 x 4 mm internal dimensions) column equipped with a pre-column (100 Å pore size, diol-coated, 5 µM particle size). The mobile phase consisted of 80 % (v/v) of 50 mM sodium phosphate buffer (pH 6.0), 15% acetonitrile and 5% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with a UV detector linked to computer data system. chromatogram of 4-methylaminoantipyrine (4-MAA) metabolites by microsomes. A reaction mixture (200 µl) including 1.25 mg/ml of microsomal protein of male wistar rats and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer, (pH 7.4). 4-Methylaminoantipyrine metabolites were extracted and analyzed by HPLC.

4-methylaminoantipyrine (50 µM) was incubated with rat liver microsomes (5 mg/ml of microsomal protein) at 37 °C for 20 min and the metabolites were analyzed by HPLC after extraction. No metabolites were seen when 4-methylaminoantipyrine and microsomes were

incubated without NADPH and with NADPH but without any incubation time (0 min). In rat liver microsomes, the metabolism of 4-methyaminoantipyrine was strongly inhibited by a concentration of 50 µM omeprazole as shown in Fig. 10, the inhibition was (65 % inhibition) and to a lesser degree by ketoconazole (37 % inhibition) and but no inhibition was detected with alpha-naphthoflavone, coumarin, quinidine and sulphaphenazole as shown in Table 12.

Table 12 Estimated % inhibition of the formation of 4-aminoantipyrine by selective chemical inhibitors added at a concentration of 50 µM. A reaction mixture (200 µl), 5 mg/ml of microsomal protein of male Wistar rats, and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37°C in 25 mM potassium phosphate buffer, (pH 7.4) 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC.

RLM			
Inhibitors	% Inhibition	K_i (mM)	IC_{50} (mM)
Omeprazole	65.90	0.04	0.05
Ketoconazole	36.60	0.14	0.77
Sulphaphenazole	(no inhibition)	-	-
Coumarin	(no inhibition)	-	-
Quinidine	(no inhibition)	-	-
Alpha-naphthoflavone	(no inhibition)	-	-

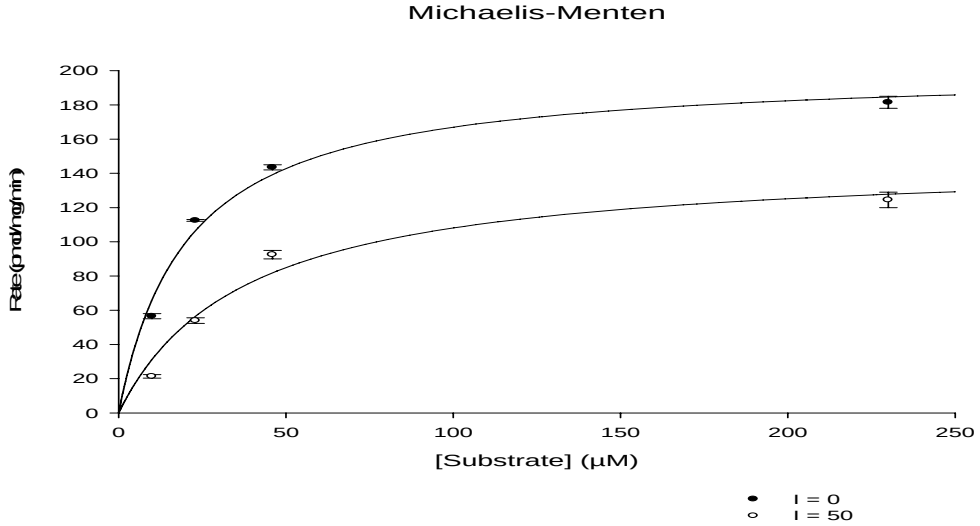

Fig. 10 Inhibition kinetic plot of metabolism of MAA with RLM by omeprazole. The reaction mixture (200 µl), contained 1.25 mg/ml of microsomal protein of male Wistar rats and 1. 0 mg/ml of NADPH. 4-methylaminoantipyrine was incubated for 20 min at 37 °C with 50 µM omeprazole in 25 mM potassium phosphate buffer, (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC.

4.2 Investigations of the metabolism of metamizole by HLM

HPLC Analysis. Typical chromatograms obtained from reference substances of 4-aminoantipyrine (4-AA, peak 1) and methylaminoantipyrine (4-MAA, peak 2) obtained after injection of 10 µmol from AA and MAA into the HPLC system are illustrated in Fig. 11. The separation was complete within 20 min. The retention time of 4-AA was 7.70 min, the retention time of 4-MAA was 11.30 min and that of the internal standard (I.S., dimethylaminoantipyrine) was 16.75 min.

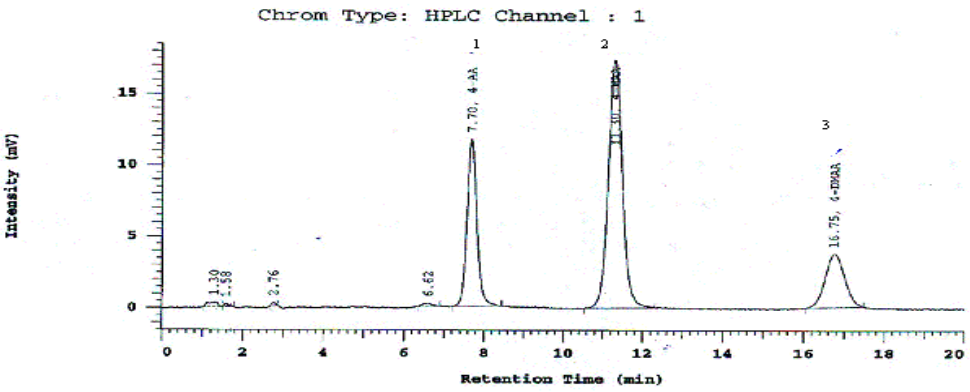

Fig. 11 Typical chromatogram of the main metabolites of metamizole obtained from standard samples. Peaks: 1: 4-AA at 7.70 min, peak 2: MAA at 11.30 min, peak 3: I.S at 16.75 min. The concentrations of 4-MAA and 4-AA were 10 µmol/l. The mobile phase consisted of 75 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0) and 25% (v/v) methanol. The flow rate was 1.0 ml/min.

The formation of 4-aminoantipyrine from 4-methylaminoantipyrine in the human liver microsomes increased in a protein-concentration and time-dependent manner. The metabolism of 4-methylaminoantipyrine was analyzed in seven preparations of human liver microsomes (HLM) with an incubation time of 20 min, and two control incubation samples, one without NADPH and one with zero incubation time.

4-Methylaminoantipyrine was incubated with human liver microsomes (2.5 mg/ml of microsomal protein, final concentration, at 37 °C) for 20 min and the metabolites were analyzed by HPLC after extraction. No metabolites were seen when 4-methylaminoantipyrine and microsomes were incubated without NADPH and also not if incubated with NADPH but with an incubation time of zero. The formation was linear at to 10 mg/ml microsomal protein and 20-min incubation time. The formation of 4-aminoantipyrine from 4-methyl-aminoantipyrine by one preparation of human liver microsomes is illustrated in a chromatogram in Fig. 12 and in dependence from substrate concentration in Fig. 13.

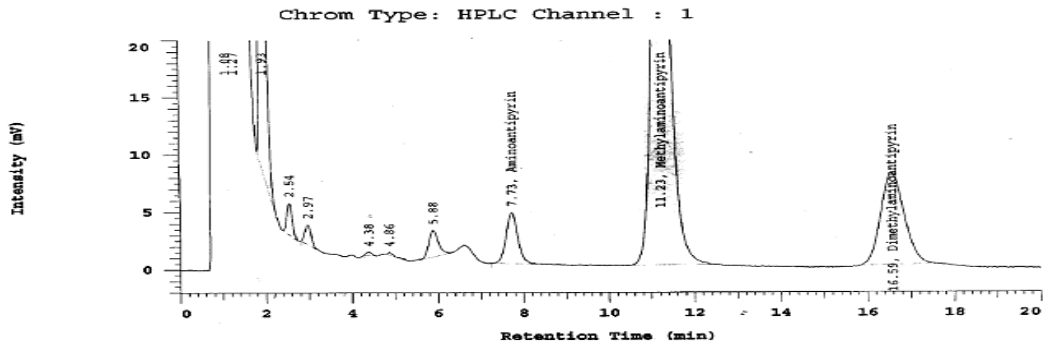

Fig. 12 HPLC-analysis of an extract of 4-methylaminoantipyrine and its metabolites after incubation with human liver microsomes. HPLC was performed with a RP-8 endcapped (5 µm) (125 x 4 mm) column equipped with a pre-column (100 Diol, 5 µM). The mobile phase consisted of 75 %(v/v) of 25 mM sodium phosphate buffer (pH 6.0) and 25%(v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with an UV detector linked to a computer data system. A reaction mixture (100 µl; 2.5 mg/ml of microsomal protein of HLM) with 1.0 mg/ml of NADPH, and 100 µmo/l of 4-methylamino-antipyrine was incubated for 20 min at 37°C in 50 mM potassium phosphate buffer (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC. Peaks: AA at 7.73 min, MAA at 11.23 min, I.S. at 16.59 min.

A maximum biotransformation rate (V_{max}) of 143 (standard deviation 30) pmol/min/mg was measured. The quantity of microsomes protein in one sample was 2.5 mg protein. The mean Michaelis-Menten constant (K_M) was 154 µmol/l with a standard deviation of 40 µmol/l as shown in Fig. 13. The intrinsic clearance was 0.92 µl/min/mg.

Table 13 Enzyme kinetic parameters for metamizole demethylation by human liver microsomes. The metamizole concentration ranged from 25 to 800 µmol/l. The protein concentration of HLM protein was 10 mg/ml.

Preparation	K_M (µM)	V_{max} (pmol/min/mg microsomal protein)	Intrinsic clearance
HL008	154	143	0.92
HL 001	251	144	0.57
HL 009	201	163	0.81
HL 010	289	147	0.51
HL 003	152	312	2.05
HL 014	228	111	0.49
Mean (standard deviation) from the 6 preparations given above	213 (54.4)	170 (71.6)	0.89 (0.59)
Liver withouth CYP2C19 (genotype *2/*2)			
HL 016	477	166	0.34
All parameters from the 7 microsomal preparations given above were derived from measurements performed in duplicates for each substrate concentration.			

In the chemical inhibition study with human liver microsomes an inhibitory effect on 4-methylaminoantipyrine N-demethylation was seen for fluvoxamine as illustrated in Fig. 12. In Table 14 further data are given showing that inhibition occurred also from co-incubation with omeprazole, moclobemide and tranylcypromine. Other selective inhibitors had smaller effects on the metabolism of metamizole in microsomal incubation (Fig. 15).

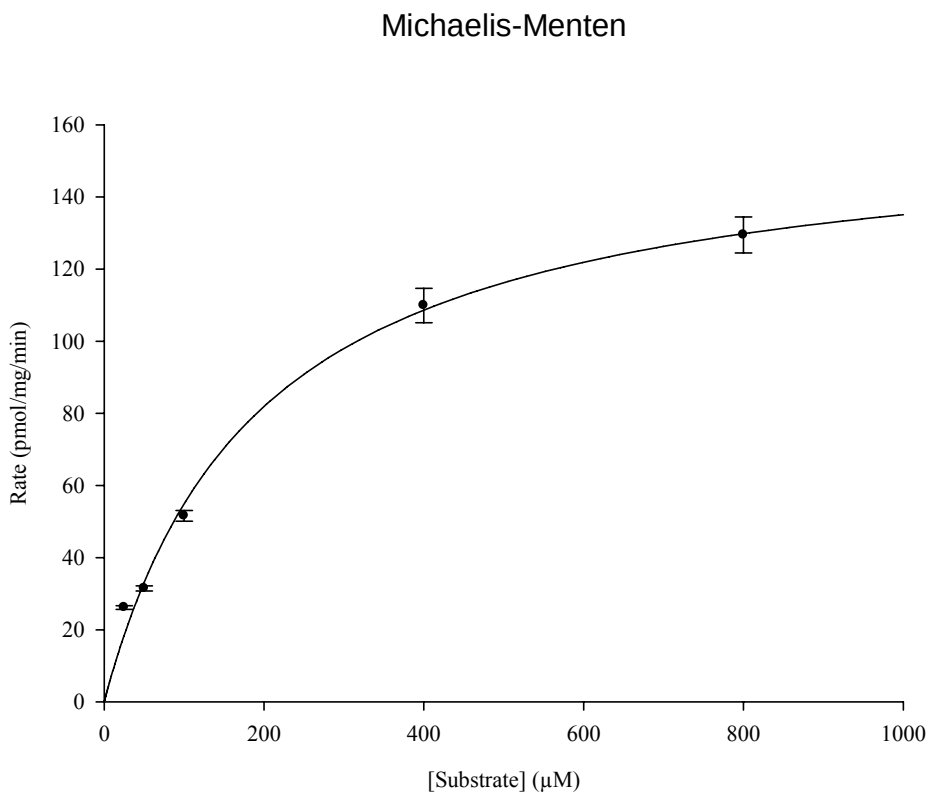

Fig. 13 Plot of the substrate concentration versus metabolite formation rate for demethylation of 4-methylaminoantipyrine by human liver microsomal preparation HLM008. The reaction mixtures (100 µl) with 2.5 mg/ml of microsomal protein, 1.0 mg/ml of NADPH. and 4-methylaminoantipyrine were incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC.

Table 14 Inhibition effects of inhibitors on 4-aminoantipyrine formation from methylaminoantipyrine. Extract from human liver microsomes obtained 20 min after incubation with 4-MAA 50 µmol/l with 50 µM from chemical inhibitors was monitored by HPLC analysis. Results are presented as average of duplicate incubations.

HLM			
Inhibitors	**% Inhibition***	**K_i (mM)**	**IC_{50} (mM)**
Fluvoxamine	57.12	0.06	0.07
Omeprazole	51.09	0.06	0.07
Tranylcypromine	50.32	0.15	0.18
Moclobemide	43.24	0.66	0.79
Coumarin	31.86	1.20	1.44
Sulphaphenazole	25.06	1.31	1.54
Ketoconazole	27.95	1.45	1.86
Alpha-naphthoflavone	18.65	3.90	4.68
Furafylline	16.09	4.01	5.41
Quinidine	11.35	6.88	8.46
* % inhibition refers to the inhibition produced by co-incubation of 50 µM 4-MAA with 50 µM of the various inhibitors compared with the incubations without inhibitor.			

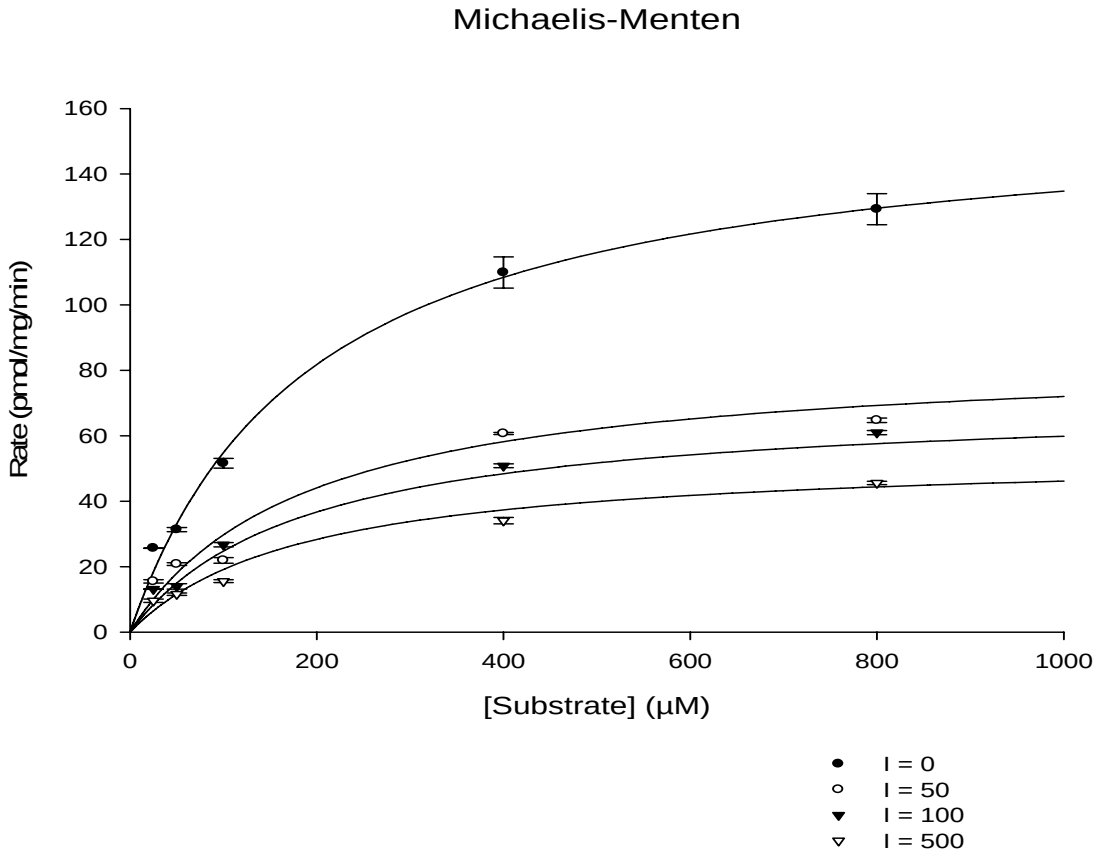

Fig. 14 Illustration of the concentration-dependent inhibition of the metabolism of 4-MAA by fluvoxamine. The reaction mixtures (100 µl) included 2.5 mg/ml of microsomal protein, 1.0 mg/ml of NADPH and 4-methylaminoantipyrine in the concentrations given in the figure as well as fluvoxamine in concentrations of 0, 50, 100 and 500 µM and it was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4).

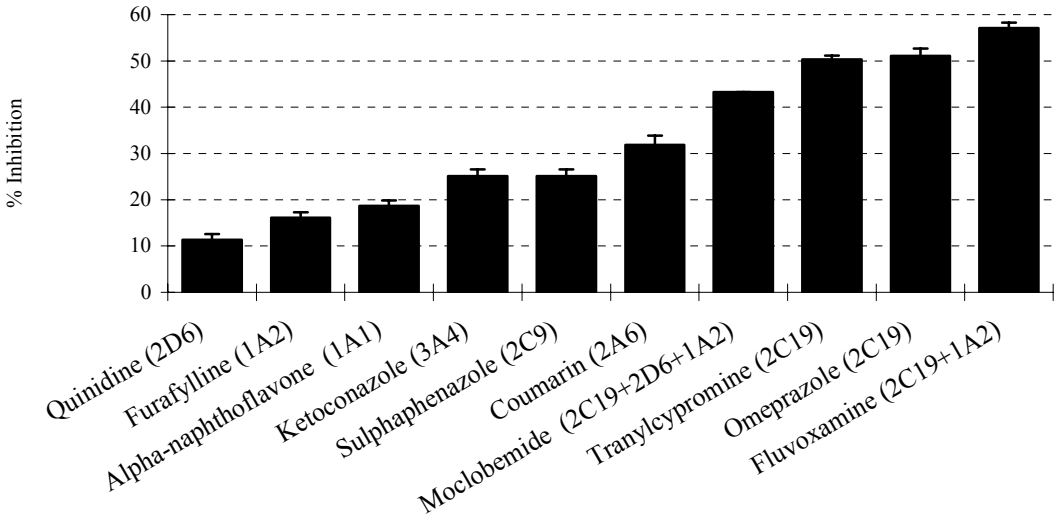

Fig. 15 Inhibition of the metabolism of 4-dimethylaminoantipyrine by characteristic inhibitors of specific CYP enzyme. Extract from human liver microsomes obtained 20 min after incubation with 4-MAA 50 µM with 50 µM from chemical inhibitors was monitored by HPLC analysis. Results are presented as average of duplicate incubations. The detailed numbers are given in Table 14.

The metabolism of 4-methylaminoantipyrine was inhibited by 57.21, 51.09 and 50.32% by a concentration of 50 µmol/l of the known CYP2C19 inhibitors fluvoxamine, omeprazole and tranylcypromine with K_i values of 0.06, 0.06 and 0.15 mM respectively.

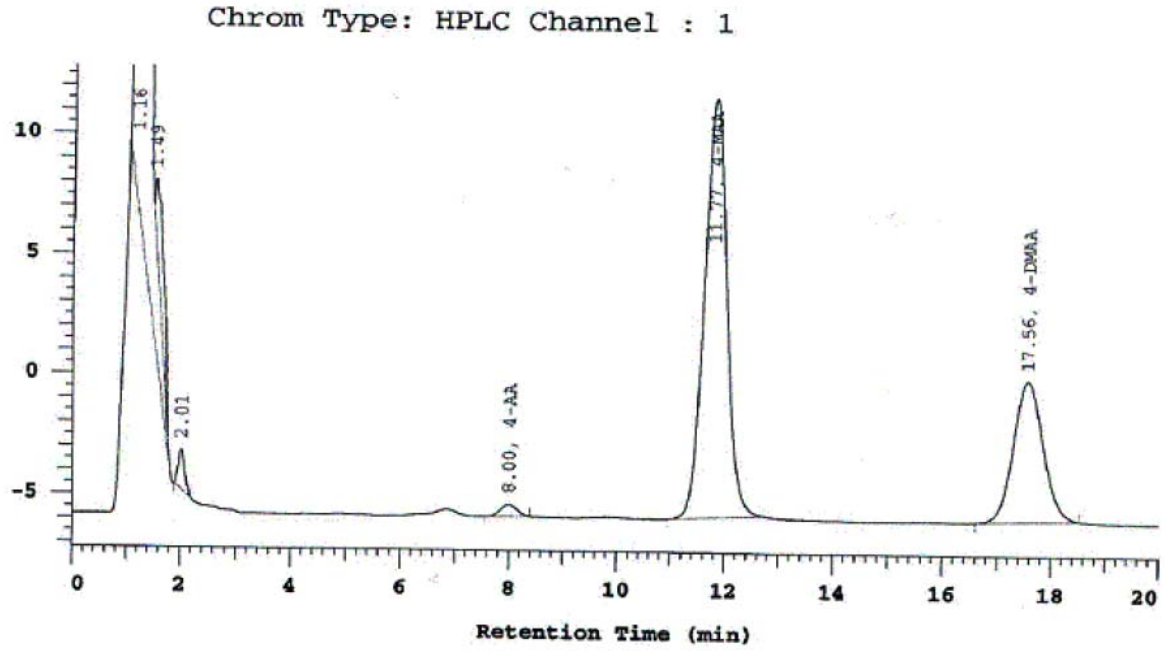

Fig. 16 Extract of 4-methylaminoantipyrine and its metabolites by HPLC with a RP-8 endcapped (5 µm) (125 x 4 mm) column equipped with a pre- column (100 Diol, 5 µM). The mobile phase consisted of 75 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0) and 25 %(v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with an UV detector linked to computer data system. The chromatogram shows 4-methylaminoantipyrine metabolites formed by the microsomes. A reaction mixture (100 µl) 2.5 mg/ml of microsomal CYP2C19 genotype (*2/*2) protein of HLM, 1.0 mg/ml of NADPH, and 200 µmol of 4-methylaminoantipyrine was incubated for 20 min at °37 in 50 mM potassium phosphate buffer, (pH 7.4.) 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC. Peaks: AA (8.00 min), MAA (11.77 min), I.S. (17.56 min).

The corresponding IC_{50} values were 0.07, 0.07 and 0.18 mM respectively. The IC_{50} values seen with coumarin, sulphaphenazole, ketoconazole, moclobemide, quinidine, alpha-naphtho-flavone and furafylline were: 0.79, 1.20, 1.36, 1.44, 3.46, 4.68, and 8.41 mM and the ki values were 0.66, 1.00, 1.13, 1.20, 2.88, 3.90 and 7.01 mM respectively (Table 14). These chemical inhibition data suggested that CYP2C19 was primarily responsible for the metabolism of 4-methylaminoantipyrine. The production of AA by microsomes CYP2C19 genotype (*2/*2, meaning expression of not any CYP2C19 enzyme) was very low as illustrated in the following HPLC chromatogramn (Fig. 16). As summarized in Table 13, mean KM for 4-MAA demethylation of microsomes expressing CYP2C19 was 213 µM (standard deviation 54.4) compared with 477 µM in the microsomes of a subject deficient in CYP2C19.

4.3 Investigations of the metabolism of metamizole by recombinant Human CYP

The enzymes kinetics of metabolism of 4-metylaminoantipyrine were finally studied by recombinant Human specific human CYP enzymes to confirm the specific CYP enzyme involved in biotransformation of 4-methyl-aminoantipyrine, which appeared to be the enzyme CYP2C19 according to the inhibition study and according to one experiment with human liver microsomes from a CYP2C19 deficient subject. Microsomes carrying only specific individual recombinant human cytochrome P450 isozymes (CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) were incubated with different concentrations of 4-MAA from 25 to 800 µmol/l in the presence of an NADPH-regenerating system at 37°C for 20 min. The substrate 4-methylaminoantipyrine was converted into 4-aminoantipyrine by all cytochrome P450 isoenzyme praparations when tested at high substrate concentration. The enzyme kinetic constants are given in (Table 15). Thus, some formation of 4-AA was observed in the incubations with CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7, whereas the high formation of 4-AA was observed only with CYP2C19 and with CYP1A2, data are given in Fig. 17, 18 and 19 respectively.

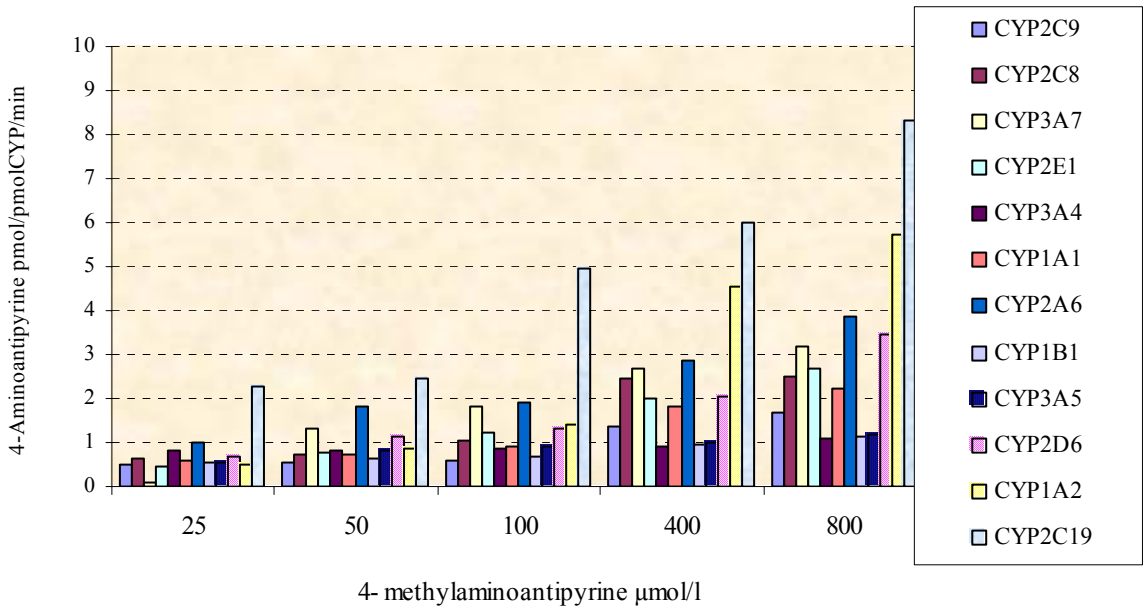

Fig. 17 Cytochrome P450 isozymes active in the in-vitro demethylation of 4-methylaminoantipyrine. 4-MAA was incubated with microsomes expressing human recombinant P450 isozymes (0.6 pmol/µl) for 20 min and the concentrations of 4-methylaminoantipyrine were 25, 50, 100, 400, 800 µmol/l. The formation of 4-aminantipyrine AA was monitored by HPLC analysis with UV detection. Results are given as means of duplicate incubations.

The formation rates of 4-aminoantipyrine (AA) with rCYP2C19 were faster than with the other P450 isozymes. Also, the highest catalytic efficiency (intrinsic clearance, V_{max}/K_m) was observed with CYP2C19, (0.077 µl/min/pmol).

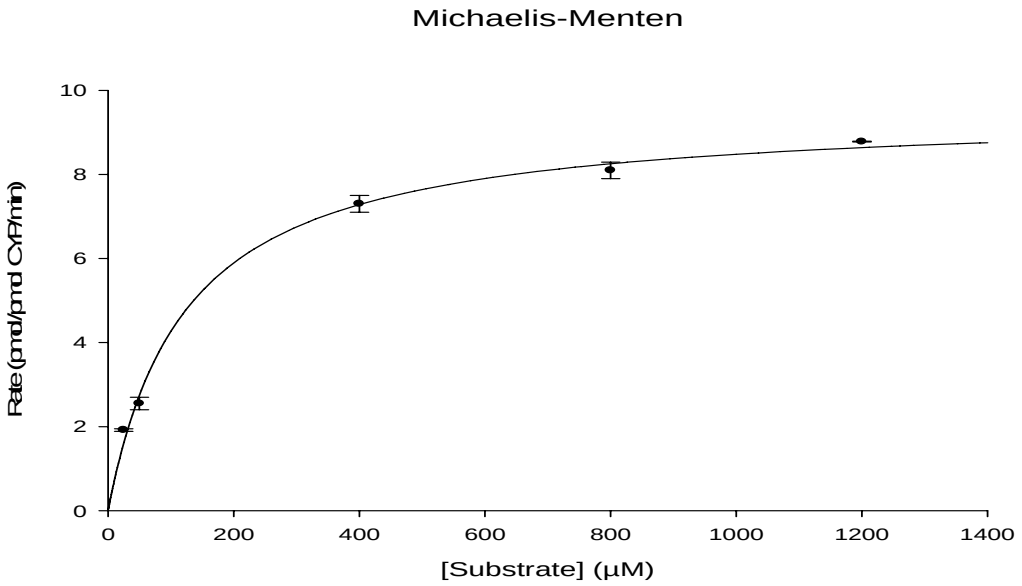

Fig. 18 Kinetic plot of demethylation of 4-methylaminoantipyrine by recombinant Human CYP2C19 enzyme. MAA was incubated with human recombinant CYP2C19 enzymes (0.6 pmol/µl) for 20 min and the concentrations of 4-methyaminoantipyrine were 25, 50, 100, 400, 800, and 1200 µmol/l.

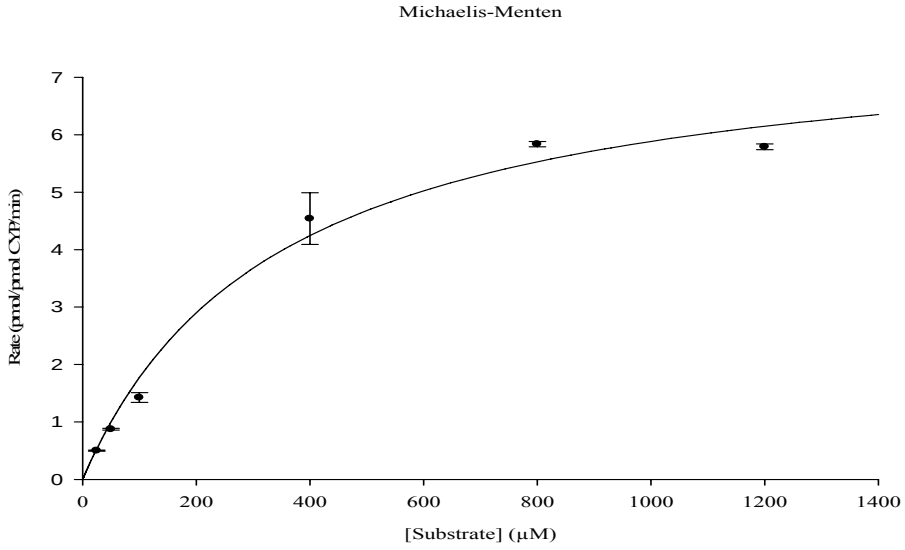

Fig. 19 Kinetic plot of demethylation of 4-methylaminoantipyrine by recombinant Human CYP1A2 enzyme. MAA was incubated with human recombinant CYP1A2 enzymes (0.6 pmol/µl) for 20 min and the concentrations of 4-methyaminoantipyrine were 25, 50, 100, 400, 800, and 1200 µmol/l.

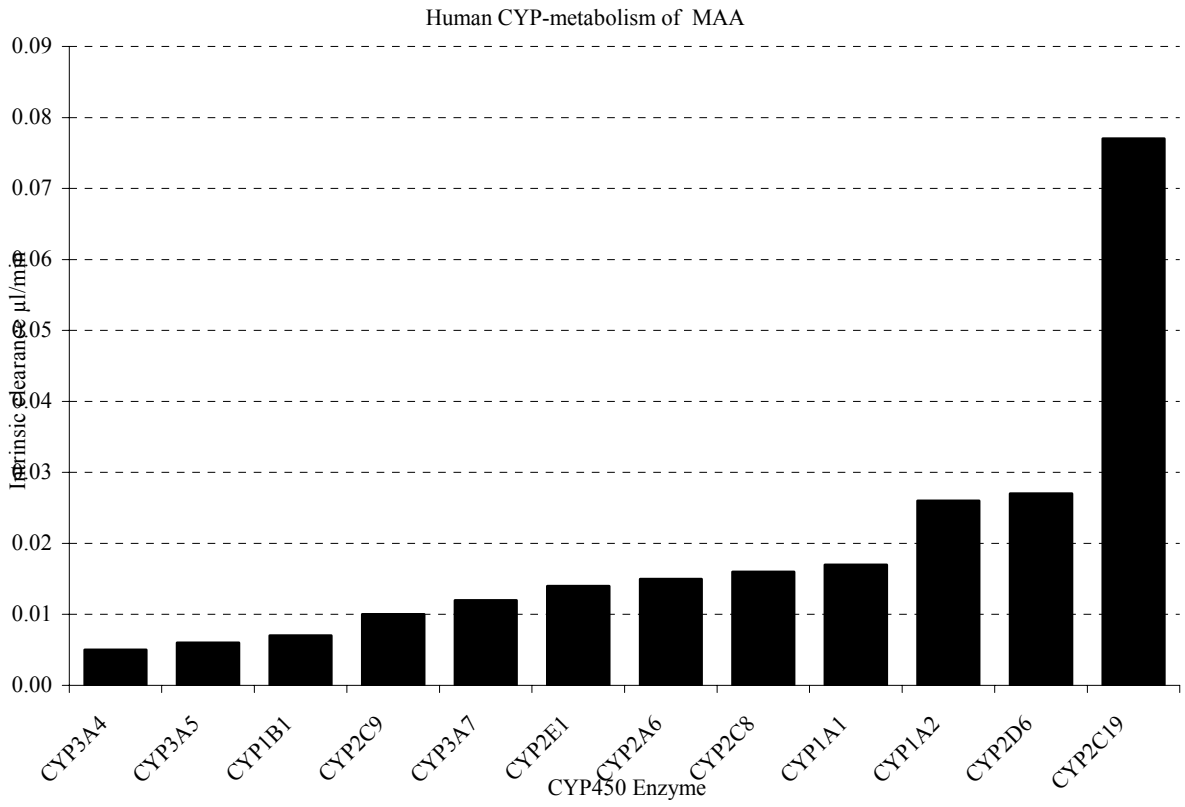

Fig. 20 Intrinsic clearance of 4-methylaminoantipyrine by specific cytochrome P450 enzymes determined after incubation of 4-MAA with microsomes expressing human recombinant cytochrome P450 isozymes (0.6 pmol/µl) for 20 min. The formation of aminantipyrine (AA) was monitored by HPLC analysis with UV detection. Results are given as means of duplicate incubation.

Table 15 Enzyme kinetic parameters for metamizole demethylation by cytochrome P450 enzymes. The metamizole concentrations was from 25 to 800 µM. The concentration of baculovirus-expressed enzymes was 0.6 pmol/µl. All data represent the mean of minimally two experiments.

Enzyme	V_{max} (pmol/pmolCYP/min)	K_m (µmol/l)	Cl_{int} (µl/pmol CYP/min)	Cl extrapolated (l/min)
CYP2C19	9.5	123	0.077	4.634
CYP2D6	3.7	138	0.027	0.269
CYP1A2	8.1	317	0.026	1.199
CYP1A1	2.6	150	0.017	0.465
CYP2C8	4	245	0.016	0.413
CYP2A6	3.8	260	0.015	0.588
CYP2E1	2.3	169	0.014	0.510
CYP3A7	2.2	177	0.012	1.790
CYP2C9	2.2	216	0.010	0.031
CYP1B1	1.4	193	0.007	0.614
CYP3A5	1.5	263	0.006	0.012
CYP3A4	1.6	315	0.005	0.009

The average immunoquantified levels of the various specific P450s in human liver microsomal samples were 25, 42, 1.2, 1.4, 6.7, 16.9, 17.9, 31.3, 2.0, 26.8, 33.6, and 96 pmol/mg proteins in human liver for the CYP2C19, CYP1A2, CYP1B1, CYP1A1, CYP2D6, CYP2C8, CYP2A6, CYP2E1, CYP3A7, CYP2C9, CYP3A5, and CYP3A4 respectively (Stormer et al., 2000a). The 4-MAA clearances extrapolated for a typical human liver based on the content of specific human CYP enzymes were 4.634, 0.269, 1.199, 0.465, 0.413, 0.588, 0.510, 1.790, 0.031, 0.614, 0.012 and 0.009 l/min given in the order of cytochrome P450 enzymes as above. In conclusion of our in vitro investigations, cytochrome P450 2C19 appeared as the primary enzyme metabolizing metamizole. The data presented here supported the results obtained from clinical studies that CYP2C19 is clinically important enzyme responsible of the metabolism of a number of therapeutic agents.

4.4 Investigations of the metabolism of Aminopyrine by HLM

Aminopyrine

HPLC Analysis. A typical chromatogram of the tested reference substances of 4-methylaminoantipyrine (4-MAA, peak 1) and 4-dimethylaminoantipyrine (4-DMAA, peak 2) measured with HPLC after injection of 20 µl from the standard solution is illustrated in Fig. 20. As illustrated, the separation was completed within 11 min. The relative order of peak retention and retention times were 5.51 min for 4-MAA, 7.07 min for 4- DMAA, and 10.38 min for the internal standard (sulfaphenazole) as given in Fig. 21.

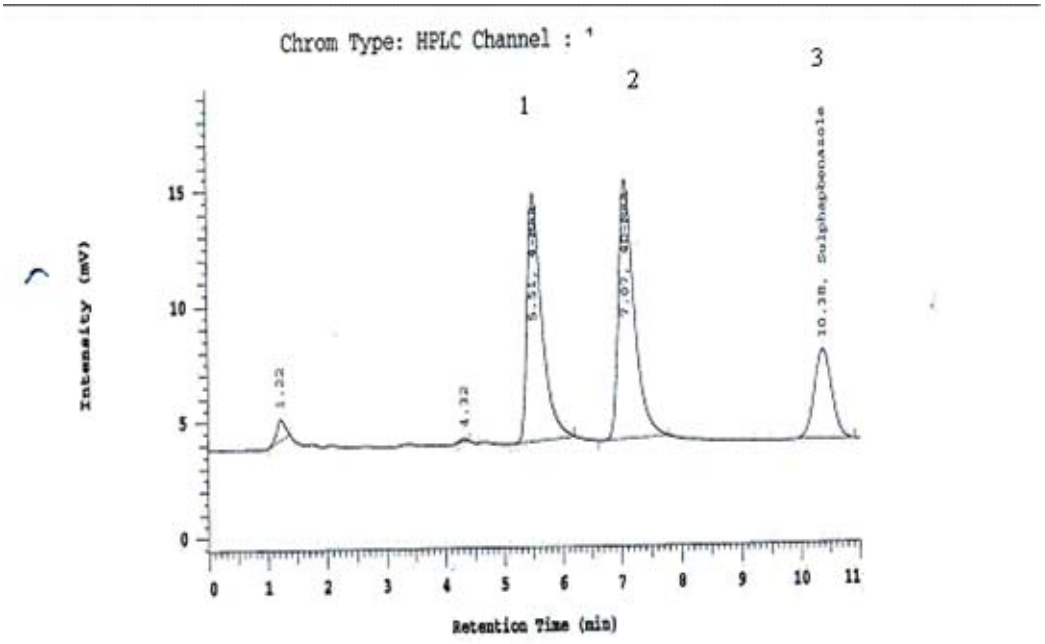

Fig. 21. Typical chromatograms of the main metabolite of aminopyrine obtained from standard samples. Peak 1: MAA (at 5.51 min) , peak 2: DMAA (at 7.07 min), peak 3: Internal standard (sulphaphenazole) at 10.38 min. The mobile phase consisted of 80 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0), 19 % acetonitrile and 1 % (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm UV detector linked to computer data system.

N-demethylation of 4-dimethylaminoantipyrine was firstly studied in human liver microsomes. The formation of 4-methylaminoantipyrine from 4-dimethylaminoantipyrine in the human liver microsomes increased in protein concentration and time dependent manners. The metabolism of 4-dimethylaminoantipyrine was analyzed in difference preparations of human liver microsomes (HLM) with many incubations with an incubation time of 20 min, and two control incubation samples, firstly without NADPH and secondly with a zero incubation time. 4-dimethylaminopyrine (200 µmol/l) was incubated with human liver microsomes 10 mg/ml of microsomal protein at 37 °C for 20 min and the metabolites were analyzed by HPLC after extraction. The metabolites were not seen when 4-dimethylaminoantipyrine and microsomes were incubated without NADPH and with NADPH but incubation time (0). Unless specified, the standard incubation mixture contained 2.5 mg/ml microsomal protein (the microsomal protein stock solution hat 10 mg/ml which was diluted 1 + 3 with the other assay components). Incubation was at 37°C for 20 min. A chromatogram showing the formation of 4-methylaminoantipyrine from 4-dimethylaminoantipyrine by human liver microsomes is shown in Fig. 22 and in dependence from substrate concentration in Fig. 23.

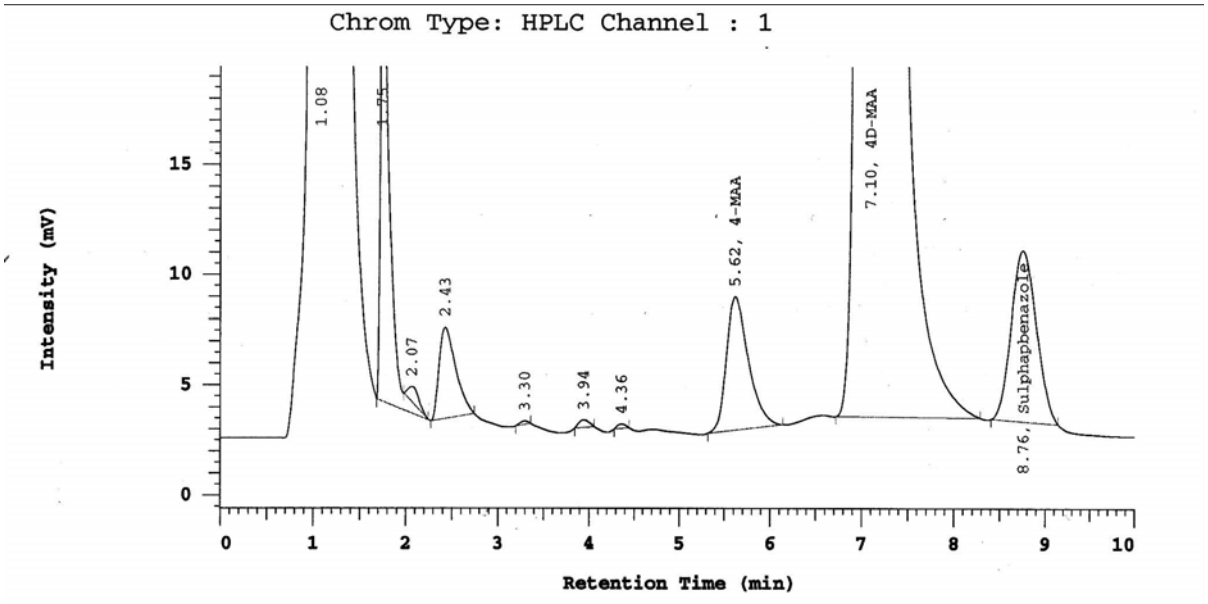

Fig. 22 Extract of aminopyrine and its metabolite analyzed by HPLC with a RP-8 endcapped (5 µm particle size) (125 x 4 mm internal dimensions) silica gel column equipped with a pre-column (100 Diol, 5 µM). The mobile phase consisted of 80 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0), 19% acetonitrile and 1% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with an UV-HPLC-detector linked to computer data system. The chromatogram shows the 4-aminopyrine metabolite 4-MAA formed by the microsomes. In this example, a reaction mixture (100 µl) with 2.5 mg/ml of microsomal protein, 1.0 mg/ml of NADPH, and 1 mM of 4-aminopyrine was incubated for 20 min at 37°C in 50 mM potassium phosphate buffer (pH 7.4). 4-aminopyrine metabolites were extracted and analyzed by HPLC. Peaks: MAA: 5.62 min, DMAA: 7.10 min, I.S: 8.76 min.

A maximum biotransformation rate (V_{max}) of 443.75 pmol/min/mg (standard deviation 20) was measured are given in Fig. 23. The quantity of microsomal protein in one sample was 2.5 mg protein. The mean Michaelis-Menten constant was K_m 472 µmol/l (standard deviation of 40) µmol/l and intrinsic clearance was 0.94 µl/min/mg (Table. 16).

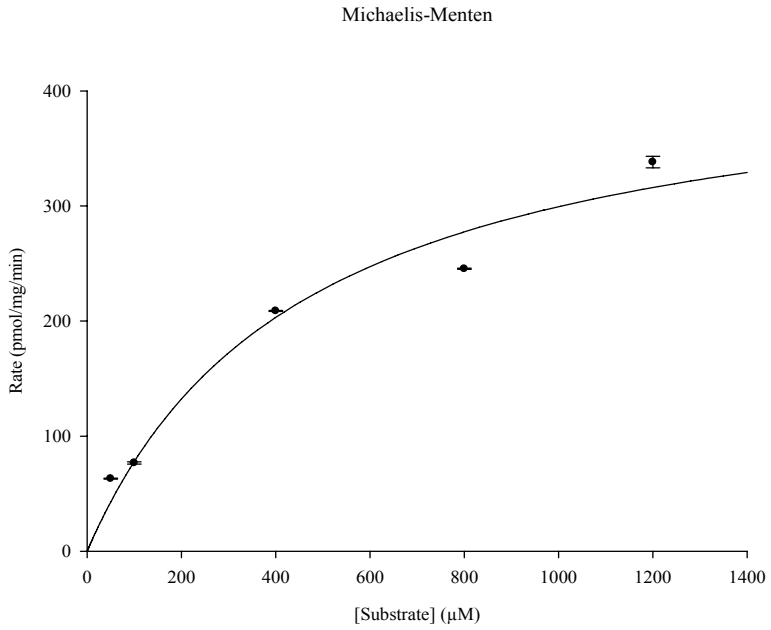

Fig. 23 Plot of the substrate concentration versus metabolite formation rate for demethylation of 4-dimethylaminoantipyrine by HLM. The reaction mixture (100 µl) included a final concentration of 2.5 mg/ml HLM protein and 1.0 mg/ml of NADPH. 4-dimethylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4). The concentrations of 4-dimethylaminoantipyrine were 25, 50, 400, 800, 1200 µmol/l 4-dimethylaminoantipyrine.

Table 16. Enzyme kinetic parameters for aminopyrine demethylation by human liver microsomes. The aminopyrine concentration range was from 25 to 800 µmol/l. The protein concentration of HLM protein was 2.5 mg/ml.

Preparation	K_M	V_{max}	Intrinsic clearance
HL 016	449	455	1.01
HL 009	472	443	0.94
HL014	431	424	0.98

The chemical inhibition study with human liver microsomes showed a significant inhibitory effect on 4-dimethylaminoantipyrine N-demethylation for quinidine and moclobemide (Table 17 and Fig. 24).

Table 17 Inhibitory effects of various CYP-specific inhibitors on 4-methylaminoantipyrine formation from 4-dimethylaminoantipyrine. The extract from human liver microsomes obtained 20 min after incubation with 4-MAA with a concentration of for instance 50 µmol/l with 50 µM from chemical inhibitors was monitored by HPLC analysis. Results are presented as average of duplicate incubations. The column with % inhibition refers to a substrate and an inhibitor concentration of 50 µM, each.

	HLM		
Inhibitors	% Inhibition	K_i (mM)	IC_{50} (mM)
Quinidine	60.01	0.05	0.06
Moclobemide	55.17	0.11	0.13
Furafylline	28.25	0.22	0.26
Alpha-naphthoflavone	13.49	0.46	0.55
Ketoconazole	8.35	0.55	0.66
Coumarin	6.19	1.10	1.33

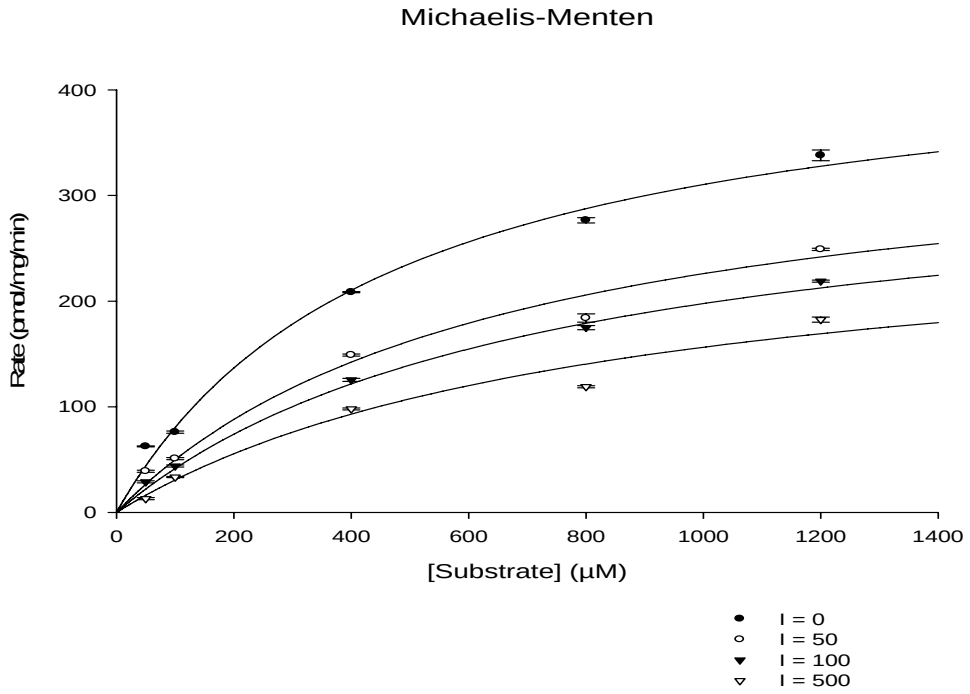

Fig. 24 Plots showing the inhibition of the metabolism of DMAA by quinidine. The reaction mixtures (100 µl) contained 2.5 mg/ml of microsomal protein of HLM and 1.0 mg/ml of NADPH and the various concentrations of 4-dimethylamino-antipyrine and were incubated for 20 min at 37°C in 25 mM potassium phosphate buffer (pH 7.4). 4-dimethylaminoantipyrine metabolites were extracted and analyzed by HPLC.

No major inhibition was observed in incubations with ketoconazole, furafylline, alpha-naphthoflavone and coumarin.

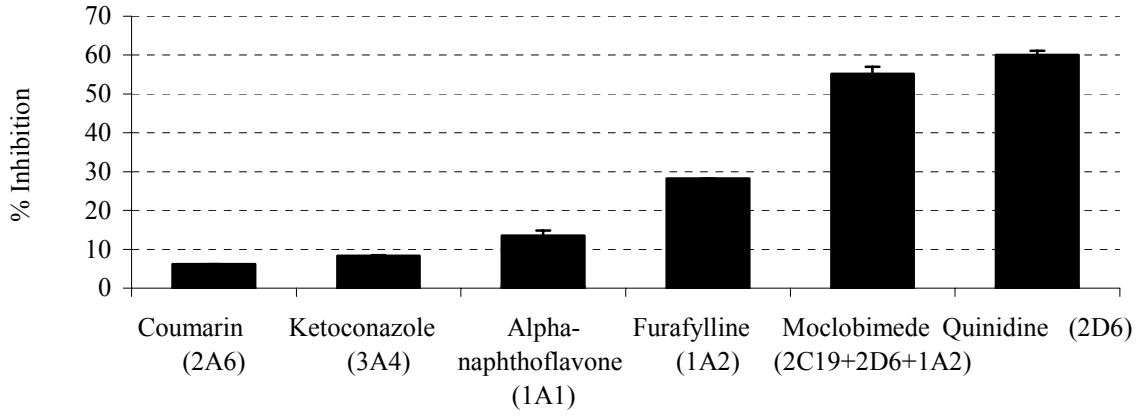

Fig. 25 Inhibition of demethylation of 4-dimethylaminoantipyrine by selective chemical inhibitors of CYP450 isoenzymes. Results are presented as arithmetic means of duplicate incubations.

The metabolism of 4-dimethylaminoantipyrine was inhibited by 60% and by 55.17 % by a concentration of 100 µmo/l of the known CYP2D6 inhibitors quinidine and moclobemide. The corresponding Ki values were 0.050 and 0.11 mM, respectively. The corresponding IC_{50} values were 0.06, and 0.13 mM respectively. The IC_{50} values seen with furafylline, alpha-naphthoflavone, ketoconazole and coumarin were 0.26, 0.55, 0.66 and 1.33 mM and the K_i values were 0.22, 0.46, 0.55 and 1.10 mM, respectively (Table 17). These chemical inhibition data suggested that CYP2D6 enzyme was primarily responsible for the N-demethylation in the metabolism of 4-dimethylaminoantipyrine.

4.5 Investigations of the metabolism of aminopyrine by recombinant Human CYP

The metabolism of 4-dimethylaminoantipyrine (4-DMAA) was studied in human liver microsomes with and without enzyme-specific inhibitors. To confirm the data concerning metabolism by CYP2D6, microsomes expressing individual recombinant human P450 isozymes (CYP1A1, CYP1B1, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2-D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) were incubated with different concentrations of 4-DMAA from 25 to 800 µmol/l at 37°C for 20 min. Formation of 4-MAA was observed in the incubations with CYP2D6, CYP2C19, CYP1A2, CYP1A1 and CYP1B1, whereas the high formation of 4-MAA was observed only with CYP2D6 as given in Fig. 26.

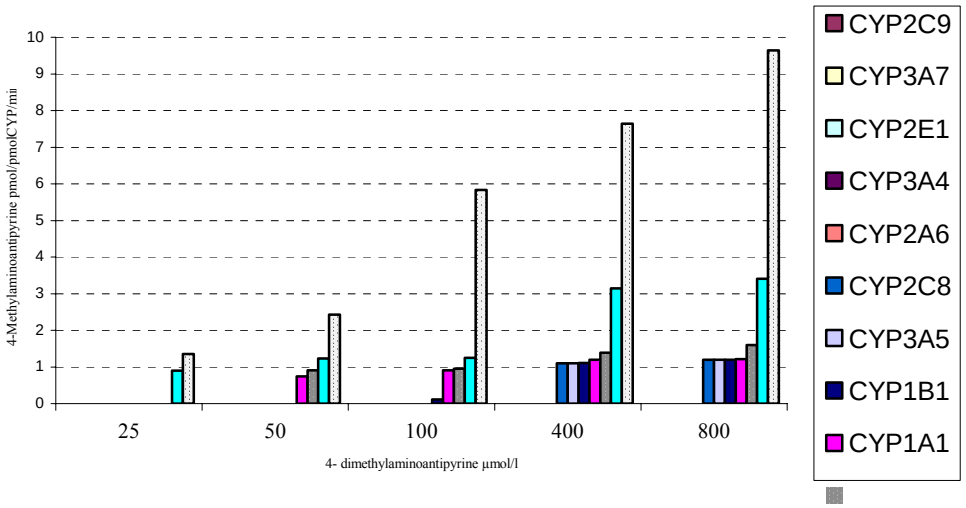

Fig. 26 Cytochrome P450 isozymes involved in the in-vitro demethylation of 4-dimethylaminoantipyrine. 4-DMAA was incubated with microsomes expressing human recombinant P450 isozymes (0.6 pmol/µl) for 20 min. The 4-dimethylaminoantipyrine concentration range varied from 50 to 800 µM.The formation of the 4-methylaminoantipyrine (MAA) was monitored by HPLC analysis with UV detection. Results are as average of duplicate incubations.

The formation rates of 4-methyaminoantipyrine (4-MAA) with rCYP2D6 were faster than with the other P450 isozymes. Also, the highest catalytic efficiency (intrinsic clearance, V_{max}/K_m) was observed with rCYP2D6 (0.011 µl/min/pmol) as illustrated in Fig. 27.

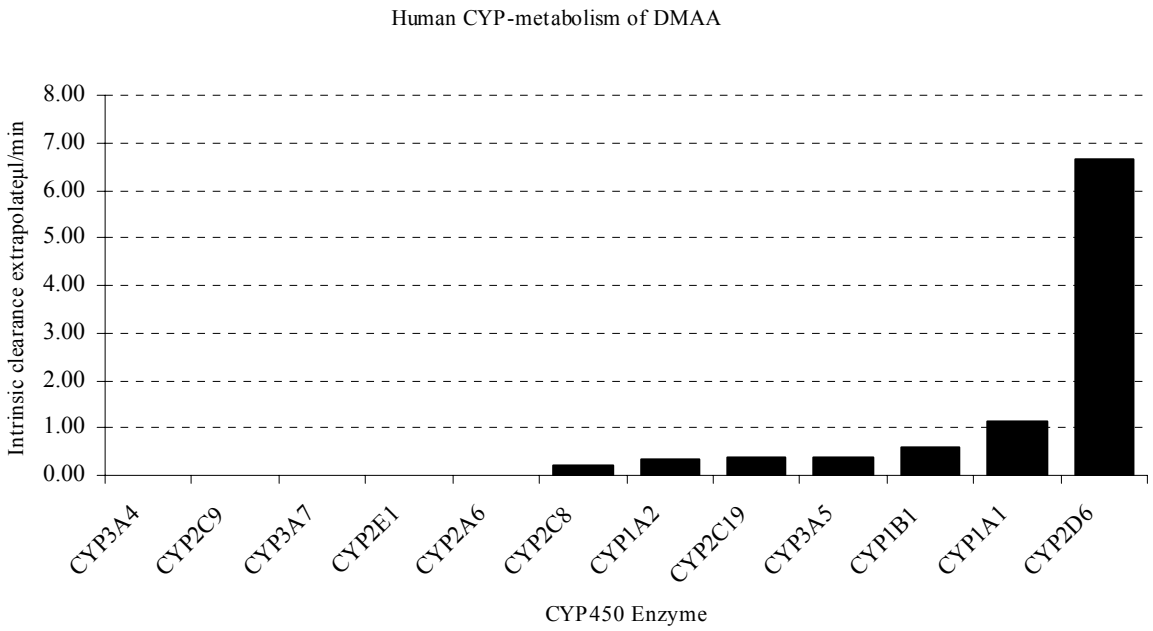

Fig. 27 Calculated intrinsic clearance of 4-dimethylaminoantipyrine by specific human cytochrome P450 enzymes after incubation of 4-DMAA with microsomes expressing human recombinant P450 isozymes (0.6 pmo/µl) for 20 min. The formation of methylaminantipyrine (MAA) was monitored by HPLC analysis with UV detection. Results are given as means of duplicate incubations.

The average immunoquantified levels of the various specific P450s in human liver microsomal samples were 6.7, 25, 1.4, 1.2, 42, 33.6, 16.9, 31.3, 26.8, 96, and 2.0 pmol/mg proteins in human liver for the CYP2D6, CYP2C19, CYP1A1, CYP1B1, CYP1A2, CYP3A5, CYP2C8, CYP2E1, CYP2A6, CYP2C9, CYP3A4 and CYP3A7 respectively(Stormer et al., 2000a). The correspondingly extrapolated clearances of specific human CYP enzymes in a typical human liver were 6.67, 0.37, 1.14, 0.60, 0.34, 0.39, 0.19, 0.0, 0.0, 0.0, 0.0 and 0.0 l/min, respectively, as given in Table 18.

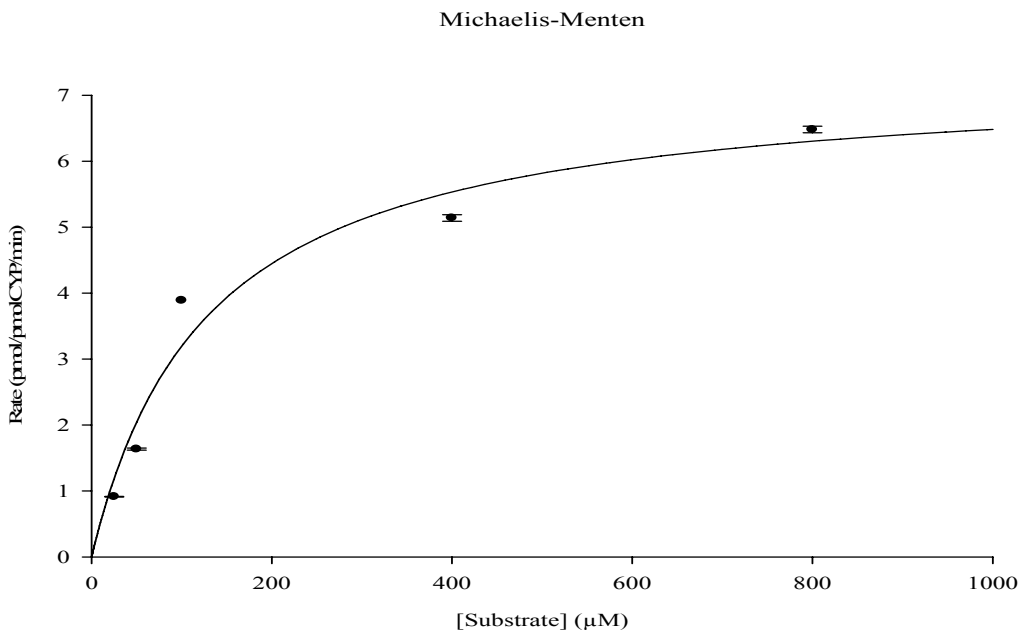

Fig. 27 Kinetic plot of demethylation of 4-dimethylaminoantipyrine by recombinant Human CYP2D6 enzyme. DMAA was incubated with human recombinant CYP2D6 enzyme (0.6 pmol/µl) for 20 min and the concentrations of 4-dimethylaminoantipyrine were 25, 50, 100, 400, 800 µmol/l. The formation of 4-methylaminantipyrine MAA was monitored by HPLC analysis with UV detection.

Table 18 Enzyme kinetic parameters for aminopyrine methylation by cytochrome P450. The aminopyrine concentration range was from 50 to 800 µM. The concentrations of baculovirus-expressed enzymes were 0.6 pmol/µl and the data represent the mean of two experiments.

4-Dimethylaminantipyrine incubation by human P450 isoforms.				
Enzyme	V_{max} (pmol/pmolCYP/min)	K_m (µmol/l)	Cl_{int} (µl/ pmol CYP/ min)	Cl extrapolate (l/min)
CYP2D6	11.9	107	0.11	6.67
CYP2C19	4.2	169	0.02	0.37
CYP1A1	5.3	219	0.02	1.14
CYP1B1	2.7	120	0.02	0.60
CYP1A2	2.1	155	0.01	0.34
CYP3A5	1.8	188	0.01	0.39
CYP2C8	6.4	1280	0.01	0.19
CYP2E1	0	0	0.00	0.00
CYP2A6	0	0	0.00	0.00
CYP2C9	0	0	0.00	0.00
CYP3A4	0	0	0.00	0.00
CYP3A7	0	0	0.00	0.00

In conclusion of our in vitro investigations, cytochrome P450 2D6 appeared as the primary enzyme metabolizing methylaminoantipyrine, and a similar effect was observed in all incubation in the present studies.

5 DISCUSSION

Aminopyrine and metamizole are used for human therapy in several European countries. In addition to the use of these drugs for postoperative pain, they are currently used in self medication. Aminopyrine played a key role in the in-vivo study of human hepatic drug metabolism, because it was widely used as a probe of liver functionality (Rodzynek et al., 1986; Lane, 1988) and functional hepatocellular mass (Krahenbuhl et al., 1989) in several diseases such as liver cirrhosis (Urbain et al., 1990), chronic hepatitis (Lashner et al., 1988), and hepatocarcinoma (Feuer, 1988; Agundez et al., 1994). In agreement with our invitro data large, interindividual differences in the capacity to detoxify such drugs have been reported (Goldberg and Brown, 1987; Lashner et al., 1988; Cotting et al., 1990; Merkel et al., 1992). Considering that the main urinary metabolites in man are acetylated and formylated as shown by others (Volz and Kellner, 1980; Cotting et al., 1990; Merkel et al., 1992; Zylber-Katz et al., 1992; Agundez et al., 1994; Agundez et al., 1995; Costa et al., 2006) who studied in-vivo the plasma concentrations of pyrazolones such as aminopyrine and metamizole or metabolism in liver disease. We have studied in vitro the oxidative biotransformation of the metamizole and aminopyrine by genetically polymorphic enzymes.

5.1 Investigations of the metabolism of metamizole by RLM and HLM

Human liver microsomes (HLM) and rat liver microsomes (RLM) are used widely to characterize the role of cytochrome P450s (P450) and other enzymes in drug metabolism. The differential centrifugation procedure used to prepare HLM is as follows: Typically, liver samples are homogenized and centrifuged at a lower force to form a crude pellet of cell debris, nuclei, peroxisomes, lysosomes, and mitochondria (premicrosomal pellet). The resulting supernatant is then centrifuged at a higher force to precipitate the microsomes. The microsomal pellet is resuspended in a final suspension buffer and is then ready for use. The complex metabolism of metamizole has been the subject of many in-vivo studies. However, the specific cytochrome P450 enzymes involved catalyzing the formation of 4-AA from 4-MAA is still not unequivocally identified.

The aim of the present study, therefore, was to validate a HPLC method suitable to study the formation of metamizole metabolites in RLM and HLM and to get insights into the CYP enzymes involved. This first study of aminopyrine using in-vitro rat liver microsomes was by (Imaoka et al., 1988) but with metamizole our investigations may be according to our best knowledge the in-vitro investigations of the metabolism of metamizole by RLM, HLM and heterologonsly expressed CYP enzymes.

The establishment of appropriate HPLC analytical methods started with the quantification of the reference substances of 4-aminoantipyrine and 4- methylaminoantipyrine. The separation was completed with in 13 min. The relative order of retention times was AA at 5.08 min and MAA at 6.72 min, as shown in Fig. 6 in results. Similar results were observed in the analysis of metamizole metabolites to study their formation in human liver microsomes (Geisslinger et al., 1996). For the incubations of metamizole with rat liver microsomes we used two different preparations of rat liver microsomes. Mean V_{max} (arithmetic mean of 5 incubations) was 201(standard deviation, SD, 42.2 pmol/mg protein/min) and mean K_M was 20.9 (SD 3.8) µmol/l are shown in Fig. 8. The corresponding intrinsic clearance was 9.61 µl/mg protein/min (SD 3.9). The quantity of microsomes protein was 1.25 mg protein. Analysis of metamizole metabolism was performed with hepatic microsomes of untreated male Wistar rats weighing between 180 to 220 g, aged 3 months, because the female rats had lower N-demethylation activity of 4-methylaminoantipyrine than male rats (Imaoka et al., 1988). The two control incubation samples without NADPH and zero incubation time are shown in Fig. 9 at the right and left part, respectively. 4-methylaminopyrine (50 µmol/l) was incubated with rat liver microsomes 5 mg/ml of microsomal protein at 37°C for 20 min and the metabolites were analyzed by HPLC after extraction. The metabolite was not seen when 4-methylaminoantipyrine and microsomes were incubated without NADPH and with NADPH but incubation time zero. This metabolite was almost not seen in an assay system with NADPH but its formation inhibited by omeprazole as shown in (Fig. 10). Omeprazole is a strong inhibitor of CYP2C19 (Imaoka et al., 1988). In rat liver microsomes, the formation of 4-aminoantipyrine from 4-methylaminoantipyrine was strongly inhibited by a concentration of 50 µM omeprazole (65% inhibition) and to a lesser degree by ketoconazole (37% inhibition) but no inhibition was detected with alpha-naphthoflavone, coumarin, quinidine and sulphaphenazole (Table 12). Therefore, our results indicated that CYP2C19 represents the main contributor to the of metabolism of metamizole in rat liver.

Human liver microsomes (HLM) are used widely to characterize the role of cytochrome P450s (P450) and other enzymes in drug metabolism. The typical differential centrifugation procedure to prepare HLM was used. Liver samples were homogenized and centrifuged at a lower force to form a crude pellet of cell debris, nuclei, peroxisomes, lysosomes, and mitochondria (premicrosomal pellet). The resulting supernatant was then centrifuged at a higher force to precipitate the microsomes. The microsomal pellets was resuspended in a final suspension buffer and was then ready for use in incubation studies. The CYP2C subfamily accounts for about 18 % of the total adults liver cytochrome P450 content (Shimada et al., 1994) the major from being CYP2C9 followed by CYP2C19 and CYP2C8 (Goldstein and de Morais, 1994; Stormer et al., 2000b). The results of the present study demonstrate that fluvoxamine, omeprazole and tranylcypromine were a competitively inhibitors of metamizole biotransformation, with k_i values of 0.06, 0.06, and 0.15 mM respectively. The metabolism of metamizole by human liver microsomes was inhibited by fluvoxamine, omeprazole and tranylcypromine by 57.12 %, 51.09 %, and 50 % respectively. A clearly less effective inhibition was observed with other P450 isoform-selective inhibitors as shown in Fig. 15. Moreover the affinity of MAA to human liver microsomes with the CYP2C19-deficient genotype *2/*2 was very low as illustrated in Table 19. The resulting formation of 4-AA was compared with the 4-AA formation from the other experiments with the CYP2C19 rapid metabolizer genotype (*1/*1) as shown in Fig. 16. Microsomes from Humans with the CYP2C19 genotype *2/*2 had low activity in other in vitro studies also (Ibeanu et al., 1998).

Table 19. The formation of 4-aminoantipyrine from 4-methylaminoantipyrine with HLM with the CYP2C19 genotype *2/*2 and HLM with the CYP2C19 genotype *1/*1. The 4-methylaminoantipyrine concentration varied from 100 to 1200 µmol/l. 2.5 mg/ml of microsomal protein was used with both preparations, with the CYP2C19 genotype (*2/*2) and with the (*1/*1) genotype. Reactions were started with 1.0 mg/ml of NADPH and were incubated for 20 min at 37°C in 50 mM potassium phosphate buffer, pH 7.4. 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC

[S]	Formation of 4-AA pmol/mg/min	
4-MAA µmol/l	HLM CYP2C19 Genotype (*1/*1)	HLM CYP2C19 Genotype (*2/*2)
100	67	52
400	205	124
800	300	256
1200	423	290

Our results from the inhibition study indicated the fluvoxamine, omeprazole and tranylcypromine are inhibitors of metamizole biotransformation. The overall results from human liver microsomes and rat liver microsomes incubation experiments with inhibitors and without inhibitors indicated that CYP2C19 was responsible primarily for the metabolism of 4-methylaminantipyrine. The strong inhibition was observed with human liver microsomes when fluvoxamine, omeprazole and tranylcypromine were used as inhibitors (Fig.15).

5.2 Investigations of the metabolism of metamizole by recombinant human CYP

To identify the CYP isozymes involved in the biotransformation of xenobiotics, in vitro methods are nowadays frequently used (Huskey et al., 1995). One way to identify the relevant enzyme in biotransformation of a given drug is to examine the effect of CYP isozyme-selective inhibitors on metabolite formation in incubations with liver microsomes expressing all cytochrome P450 enzymes. Another way is to use isolated heterologously expressed enzymes. To determine in our case the specific CYP enzymes involved in biotransformation of the metamizole primary metabolite 4-methylaminoantipyrine, microsomes expressing individual recombinant human CYP (CYP1A2, CYP1A1, CYP1B1, CYP2C19, CYP2A6, CYP2D6, CYP3A4, CYP3A5, CYP3A7, CYP2C8, CYP2C9 and CYP2E1) were incubated with six different concentrations from 4-methylaminoantipyrine in the presence of an NADPH-regenerating system at 37°C for 20 min. Under these conditions the highest formation rate of 4-aminoantipyrine was observed with CYP2C19 (Fig. 17). The formation rates of 4-aminoantipyrine with CYP2C19 were faster than with the other P450 isozymes. Also, affinity was the highest among all enzymes tested with a K_m of 123 µml/l (Table 15). And the extrapolated clearances via specific human CYP2C19, CYP1A2, CYP1B1, CYP1A1, CYP2D6, CYP2C8, CYP2A6, CYP2E1, CYP3A7, CYP2C9, CYP3A5, and CYP3A4 enzymes were 4.634, 0.269, 1.199, 0.465, 0.413, 0.588, 0.510, 1.790, 0.031, 0.614, 0.012 and 0.009 l/min, respectively. Moreover the enzyme CYP2C19 had the highest catalytic efficiency (intrinsic clearance, V_{max}/ K_m) of measurements (Fig. 20). The results from the contribution of individual P 450 isozymes were compared with the inhibition study of metamizole with human liver microsomes and rat liver microsomes.

The data presented here supported the results obtained from clinical studies that CYP2C19 is clinically important enzyme responsible of the metabolism of a number of therapeutic agents (Blaisdell et al., 2002) but the effect of CYP2C19 in metabolism of 4-methylaminoantipyrine was not known before. Historically, the prototypic probe drug to test for CYP2C19 activity was S-mephenytoin, with the S-mephenytoin to R-mephenytoin ratio being an important in vivo indicator for that activity (Blaisdell et al., 2002) and it might be a good idea in the future to compare that ratio with the metamizole pharmacokinetics in humans. More recently, the cytochrome P450 enzyme 2C19 was identified as an important enzyme of the cytochrome P450 superfamily being responsible for the biotransformation and elimination of many commonly prescribed drugs including anticonvulsants, antidepressants, the antimalaria-drug proguanil, and all currently medically used proton pump inhibitors (Goldstein and de Morais, 1994; Streetman et al., 2000). It is interesting that our study adds the analgesic and antipyretic drug metamizole to that list of CYP2C19 substrates.

5.3 Investigations of the metabolism of aminopyrine by HLM

This study started with establishment of HPLC analysis based on the reference substances 4-methylaminoantipyrine and 4-dimethylaminoantipyrine (aminopyrine). The separation is illustrated in Fig. 21 in results. The metabolism of 4-dimethylaminoantipyrine was analyzed in different preparations of human liver microsomes (HLM) with many incubations with an incubation time of 20 min and two control incubation samples without NADPH and zero time of incubation. 4-dimethylaminopyrine (200 µmol/l) was incubated with human liver microsomes with a microsomal protein concentration of 2.5 mg/ml at 37 °C for 20 min and the metabolite 4-methylaminoantipyrine was analyzed by HPLC after extraction. The metabolites were not seen when 4-dimethylaminoantipyrine and microsomes were incubated without NADPH and also not in incubations with NADPH but with and incubation time of zero. The kinetics of 4-dimethylaminoantipyrine *N*-demethylation in the human liver microsomes is illustrated in Fig. 23 in the results. A maximum biotransformation rate (Vmax) of 443.75 pmol/min/mg (standard deviation 20) was measured. The mean Michaelis-Menten constant was 472.25 µmol/l (40 µmol/l standard deviation) and intrinsic clearance was 0.94 µl/min/mg. K_m In the chemical inhibition study with human liver microsomes, the drugs quinidine and moclobemide exhibited an inhibitory effect on 4-dimethylaminoantipyrine demethylation.

Quinidine is known to be a specific inhibitor of the enzyme CYP2D6 at low concentrations whereas moclobemide is an inhibitor of CYP2C19 and CYP2D6 and slightly also CYP1A2 (Baker et al., 1999). The metabolism of 4-dimethyl-aminoantipyrine was inhibited by 60% and 55% by a concentration of 100 µmo/l of the known CYP2D6 inhibitors quinidine (Fig. 24) and moclobemide and with Ki values of 0.050 and 0.11 mM respectively. A similar value of K_i of quinidine was observed (0.03) in the similar study (Smith and Jones, 1992) with the test substrate alprenolol. The corresponding IC_{50} values were 0.06, and 0.13 respectively. The IC_{50} value seen with furafylline, alpha-naphthoflavone, ketoconazole and coumarin were 0.26, 0.55, 0.66 and 1.33 and the ki values were 0.22, 0.46, 0.55 and 1.10 mM respectively (Table 17). These chemical inhibition data suggested that CYP2D6 was primarily responsible for the metabolism of 4-dimethylaminoantipyrine since quinidine is a highly potent selective inhibitor of CYP2D6 (Abraham et al., 2001). Also in other studies for instance (Brachtendorf et al., 2002), the inhibition data set suggests that CYP2D6 is the high affinity and CYP2 C19 is the low affinity binding site, respectively, for another drug, namely maprotiline. The data presented are inter preted based on the results obtained from clinical studies, which showed the quinidine is the most potent inhibitor of enzyme CYP2D6 (Lennard, 1990; Smith and Jones, 1992) (Abraham et al., 2001).

5.4 Investigations of the metabolism of aminopyrine by recombinant Human CYP

Aminopyrine N-demethylation activity has been studied in humans mainly by use of the aminopyrine breath test, although in vitro studies remain to be done (Chengelis, 1988). Therefore we performed our complete in-vitro study on the metabolism of aminopyrine by human liver microsomes and specific human cytochrome P450 enzymes. Some formation of the 4-DMAA metabolite 4-MAA was observed in the incubations with CYP2D6, CYP2C19, CYP1A2, CYP1A1 and CYP1B1, and the highest formation of 4-MAA was observed with CYP2D6 as shown in Fig. 26 in the results. The average of immuno-quantified levels of the various specific P-450s in human liver microsomal samples were 6.7, 25, 1.4, 1.2, 42, 33.63, 16.85, 31.29, 26.82, 96, and 2.0 pmol/mg proteins in human liver for the CYP2D6, CYP2C19, CYP1A1, CYP1B1, CYP1A2, CYP3A5, CYP2C8, CYP2E1, CYP2A6, CYP2C9, CYP3A4 and CYP3A7 respectively (Shimada et al., 1994; Mizutani, 2003), and the extrapolated

clearances via the specific human CYP enzymes were 6.67, 0.37, 1.14, 0.60, 0.34, 0.39, 0.19, 0.0, 0.0, 0.0, 0.0 and 0.0 l/min, respectively, as shown in Fig. 27.

In conclusion of our in-vitro investigations, cytochrome P450 2D6 appeared to be the primary enzyme metabolizing 4-dimethylaminoantipyrine. And concerning CYP2D6 a similar and consistent effect was observed in all incubations in the present studies, namely with chemical inhibitors and with the isolated enzymes as well. Since CYP2D6 is responsible for the metabolism of many commonly used drugs, this may result may mean that the so-called poor metabolisers of substrates of CYP2D6, which are about 7% in Caucasian populations (see introduction) are at high risk for side effects of antipyrine (aminopyrine). The results obtained here on the impact of CYP2D6 for the biotransformation and elimination of analgesic-antipyretic drugs complements the results of many other studies showing that many antiarrhythmics, ß- receptor blockers, neuroleptics, anti-depressants, tamoxifen, and codeine are metabolized by CYP2D6 (Allegaert et al., 2005; Komura and Iwaki, 2005). Our results are in slight contrast with the data of (Niwa et al., 1999) who concluded that CYP2C19 is the most efficient enzyme in demethylation of aminopyrine and that CYP2C8 and CYP2D6 may also be involved.

However, Niwa et al. did not specifically measure the metabolite formed but they only quantified the reaction by the formaldehyde production and thus, it is not clear which of the two aminopyrine demethylations they really measured. Altogether, these findings suggest that CYP2C19 and CYP2D6 are clinically important enzymes responsible for the biotrans-formation (metabolism) of a number of therapeutic agents belonging to the class of analgesic-antipyretic drugs.

5.5 Clinical Implications and Conclusion

Metamizole and aminopyrine are usually used episodically for treatment of acute pain and fever, although prolonged use for musculokeletal pain or the control of pain in patients with cancer is also practised (Rodriguez et al., 1994) and (Planas et al., 1998). The drugs are widely used in some countries, while in others (for instance in Sweden) they have been banned or restricted because of the risk of adverse effects, notably agranulocytosis (Zylber-

Katz et al., 1995). However, it have been estimated by the an international study of agranulocytosis and aplastic anemia that the excess risk of agranulocytosis associated with eny metamizole and aminopyrine exposure in one treatment week is 1.1 cases per million user (Levy et al., 1995).

The same studies did not find an association between aplastic anaemia and metamizole and aminopyrine use, but only for agranulocytosis. The data of this study indicates that highly polymorphic enzymes are involved in biotransformation of these antipyretic drugs. As mentioned earlier, about 3% of most population do not have CYP2C19 activity and about 7% of many population do not have CYP2D6 activity. It is an interesting hypothesis that these slow metabolizers have a high risk for the adverse effect but this was never tested in clinical studies.

6 SUMMARY

Two formerly very frequently used analgesic drugs or active metabolites of analgesic drugs, namely 4-methylaminoantipyrine (derived from metamizole) and 4-dimethylaminoantipyrine (also termed antipyrine) were studied here concerning the specific enzymes involved in their oxidative biotransformation by genetically polymorphic and non-polymorphic enzymes. The main conclusions from this investigation are:

- In vitro metabolism of methylaminoantipyrine to 4-aminoantipyrine was characterized by a K_m of 20.9 µmol/l and a V_{max} of 201 pmol/mg/min in rat liver microsomes (1.25 mg/ml protein). Strongest inhibition of methylaminoantipyrine demethylation in rat liver microsomes was achieved with omeprazole with an IC_{50} of 0.05 mM

- In human liver microsomes, mean K_m and V_{max} were 154 µmol/l and 143 pmol/mg/min, respectively (2.5 mg/ml protein). Strong inhibition of these reactions was observed after co-incubation with omeprazole (CYP2C19), fluvoxamine (CYP2C19 and CYP1A2), and tranylcypromine (CYP2C19) with IC_{50} values of 0.07, 0.07 and 0.18 mmol/l, respectively.

- Some formation of 4-aminoantipyrine from methylaminoantipyrine was observed in the incubations with recombinant CYP2C19, CYP2D6, CYP1A2, CYP2C8, CYP2A6, CYP1A1, CYP1B1, CYP3A4, CYP3A5, CYP3A7, CYP2C9 and CYP2E1 but the highest formation was observed with CYP2C19. Intrinsic clearances were 0.077 (CYP2C19), 0.027 (CYP2D6), 0.026 (CYP1A2), 0.017 (CYP1A1) and 0.016 (CYP2C8) µl/min/pmol CYP, respectively.

- It is concluded that CYP2C19 is the most important cytochrome P450 enzyme involved in metamizole metabolism but the enzymes CYP2D6 and CYP1A2 may also be involved.

- In human liver microsomes, mean K_m and V_{max} for the demethylation of dimethylaminoantipyrine (Aminopyrine) to methylaminoantipyrine were 472 µmol/l and 443 pmol/mg/min, respectively (2.5 mg/ml protein). S inhibition of these reactions was observed after co-incubation with quinidine (a prototypic CYP2D6 inhibitor), and moclobemide.

- The formation of 4-methylaminoantipyrine from dimethylaminoantipyrine was observed in the incubations with recombinant CYP2D6, CYP2C19, CYP1A1, CYP1B1, CYP1A2, CYP3A5 and CYP2C8, but the highest formation was observed with CYP2D6 with an intrinsic clearance of 0.11 µl/pmol CYP/min. Intrinsic clearances via CYP2C19, CYP1A1 and CYP1B1 were significantly lower with values of 0.02 µl/pmol CYP/min for all these three enzymes.

- In slight contrast to earlier data of Niwa et al, we concluded that CYP2D6 may be the most important enzyme responsible for the N-demethylation of 4-dimethyl-aminoantipyrine (aminopyrine) to 4-methylaminoantipyrine in human hepatic biotransformation.

Altogether, these findings suggest that CYP2C19 and CYP2D6 are medically important enzymes responsible for the metabolism of therapeutic agents on the analgesic-antipyretic drugs, metamizole and aminopyrine and the role of the genetic polymorphisms in the genes coding for these enzymes for adverse effects should be further studied.

7 FUTURE PERSPECTIVES

The work presented in this thesis is part of clinical pharmacology studies and the clinical of pharmacology is the application of pharmacodynamics and pharmacokinetics to patients with diseases and nowadays pharmacogenetics plays an important role in this therapeutic research. The clinical pharmacologists are using data from the human genome project to determine how and why individuals respond differently to drugs and pharmacogenetics is one of the fields of clinical pharmacology, which studies how genetic factors influence drug response. Today, the relationship between dosage requirements and pharmacogenetic polymorphisms in drug metabolizing enzymes are best substantiated for cytochromes such as CYP2D6 (anti-depressants, antipsychotics, analgesics) (Kirchheiner et al., 2004; Lotsch et al., 2004; Ingelman-Sundberg, 2005) , CYP2C9 (warfarine, antidiabetics) (Kirchheiner et al., 2004) CYP2C19 (diazepam, proton pump inhibitors) (Goldstein, 2001; Desta et al., 2002). CYP2C19 is clinically important enzyme responsible of the metabolism of a number of therapeutic agents (Blaisdell et al., 2002). CYP2 family plays a dominant role in the metabolic elimination of more xeno and endobiotics than anyother biotransformation. The CYP2C subfamily in human consists of four highly homologous genes: CYP2C8, CYP2C9, CYP2C18 and CYP2C19. CYP2C accounts for about 17-20 % of the human total liver CYP content. CYP2C9 is the main CYP2C in human liver, followed by CYP2C8 and CYP2C19 (Edwards, 1998). I was interested to investigate the impact of CYP2 genetic polymorphisms for human on other drugs of analgesic-antipyretic drugs, and the contribution of CYP2C19, CYP2D6 and CYP1A2 on the in-vivo hepatic metabolism of metamizole and aminopyrine with different sources of cytochrome P450 should be compared between in-vitro and in-vivo methods in hepatic metabolism. This is also quite interesting because aminopyrine and metamizole have been widely used as a probe of liver functionality (Feuer, 1988). In addition, recently there is growing interest in the use of metamizole and aminopyrine metabolism as indicator of the outcome of hepatic transplantation.

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